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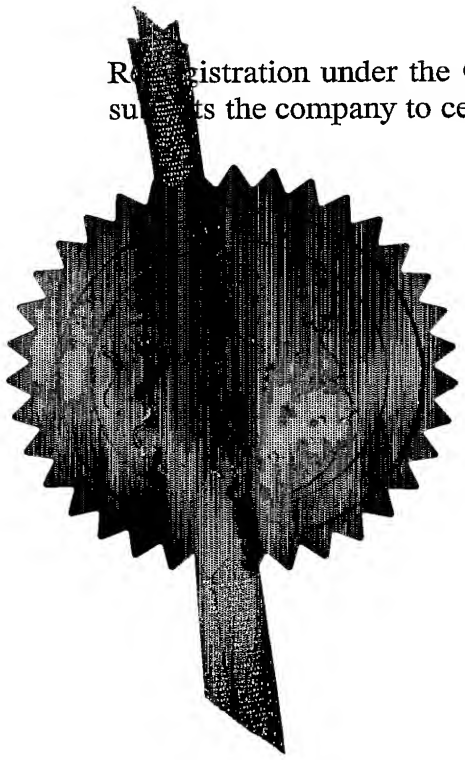

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2. Patent application number *(The Patent Office will fill in this part)* — 0317944.7 — 31 JUL 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — Gentronix Limited
The Fairbairn Building
72 Sackville Street
Manchester, M60 1QD

   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

8225385001

4. Title of the invention — GENOTOXIC TESTING

5. Name of your agent *(if you have one)* — Marks & Clerk

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — 43 Park Place
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GENOTOXIC TESTING

The present invention relates to improved methods for detecting agents that cause or potentiate DNA damage and to molecules and transformed cells that may be usefully employed in such methods.

DNA damage is induced by a variety of agents such as ultraviolet light, X rays, free radicals, methylating agents and other mutagenic compounds. These agents may cause damage to the DNA that comprises the genetic code of an organism and cause mutations in genes. In microorganisms such mutations may lead to the evolution of new undesirable strains of the microorganism. For instance, antibiotic or herbicide resistant bacteria may arise. In animals these mutations can lead to carcinogenesis or may damage the gametes to give rise to congenital defects in offspring.

These DNA damaging agents may chemically modify the nucleotides that comprise DNA and may also break the phosphodiester bonds that link the nucleotides or disrupt association between bases (T-A or C-G). To counter the effect of these DNA damaging agents cells have evolved a number of mechanisms. For instance the SOS response in E. coli is a well characterised cellular response induced by DNA damage in which a series of proteins are expressed, including DNA repair enzymes, which repair the damaged DNA.

There are numerous circumstances when it is important to identify what agents may cause or potentiate DNA damage. It is particularly important to detect agents that cause DNA damage when assessing whether it is safe to expose a person to these agents. For instance a method of detecting these agents may be used as a mutagenesis assay for screening compounds that are candidate medicaments, food additives or cosmetics to assess whether or not the compound of interest induces DNA damage. Alternatively methods of detecting DNA damaging agents may be used to monitor for contamination of water supplies with pollutants that contain mutagenic compounds.

Various methods, such as the Ames Test, for determining the toxicity of an agent are known but are unsatisfactory for a number of reasons. For instance,

incubation of samples can take many days when it is often desirable to obtain genotoxic data in a shorter time frame. Furthermore, many known methods of detecting DNA damage (including the Ames Test and related methods) assay lasting DNA damage, as an endpoint, either in the form of misrepaired DNA (mutations and recombinations) or unrepaired damage in the form of fragmented DNA. However most DNA damage is repaired before such an endpoint can be measured and lasting DNA damage only occurs if the conditions are so severe that the repair mechanisms have been saturated.

An improved genotoxic test is disclosed in WO 98/44149. This specification concerns recombinant DNA molecules comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein. Such DNA molecules may be used to transform a cell and such cells used in a genotoxic test for detecting for the presence of an agent that causes or potentiates DNA damage. The cells may be subjected to an agent and the expression of the light emitting reporter protein from the cell indicates that the agents cause DNA damage.

The genotoxic tests described in WO 98/44149 detect the induction of repair activity that prevents an endpoint being reached. The method described in WO 98/44149 may therefore be used to detect for the presence of DNA damaging agents.

WO 98/44149 describes a number of useful genetic constructs that may be used to tranform a cell such that it may be used in a genotoxic test. One such construct is yEGFP-444 (illustrated in Figure 12 of WO 98/44149) and the present invention is based upon a novel type of construct that was identified during developmental work carried out with yEGFP-444.

According to a first aspect of the invention, there is provided a recombinant vector comprising a recombinant DNA molecule comprising a RNR regulatory element operatively linked to a DNA sequence that encodes a light emitting reporter protein and a DNA vector characterised in that the vector comprises an origin of replication; at least one selectable maker; and when used to transform a cell, does not alter the sensitivity of the cell to geneticin.

According to a second aspect of the invention, there is provided a cell containing a recombinant vector in accordance with the first aspect of the present invention.

According to a third aspect of the present invention, there is provided a method of detecting for the presence of an agent that causes or potentiates DNA damage comprising subjecting a cell in accordance with the second aspect of the present invention to an agent and monitoring the expression of the light emitting reporter protein from the cell.

By "RNR regulatory element" we mean a DNA sequence that is the natural regulator of an RNR gene.

By "operatively linked" we mean that the regulatory element is able to induce the expression of the reporter protein.

By "reporter protein" we mean a protein which when expressed in response to the regulatory element of the DNA molecule of the invention is detectable by means of a suitable assay procedure.

The method of the third aspect of the invention represents a novel cost-effective genotoxicity screen, that may be used to provide a pre-regulatory screening assay for use by the pharmaceutical industry and in other applications where significant numbers of compounds need to be tested. It provides a higher throughput and a lower compound consumption than existing eukaryotic genotoxicity assays and is sensitive to a broad spectrum of mutagens and, importantly, clastogens.

The method of the third aspect of the invention is suitable for assessing whether or not an agent may cause DNA damage. It is particularly useful for detecting agents that cause DNA damage when assessing whether it is safe to expose a person to DNA damaging agents. For instance, the method may be used as a mutagenesis assay for screening whether or not known agents, such as candidate medicaments, foodstuffs or cosmetics, induce DNA damage. Alternatively the method of the

invention may be used to monitor for contamination of water supplies with pollutants containing DNA damaging agents.

The method of the third aspect of the invention may equally be used for assessing whether an agent may potentiate DNA damage. For example, certain agents can cause DNA damage by inhibiting DNA repair (for instance by preventing expression of a repair protein) without directly inflicting DNA damage. These agents are often known as co-mutagens and include agents such as lead.

The present invention is based upon work conducted on the vectors disclosed in WO 98/44149. The inventors have found that a spontaneous rearrangement of the known vector pWDH445 occurred that resulted in a brighter reporter signal. They therefore conducted experiments to characterise this fortuitous mutation (see Example 1) and then went on to design recombinant vectors according to the first aspect of the present invention that comprise a RNR regulatory element in place of RAD54

The vector pWDH445 is illustrated in figure 5. The inventors have found that, surprisingly, deletion of the *kan*MX3 gene function (i.e. removing geneticin resistance) from the pWDH445 vector results in a vector capable of expressing the reporter protein (GFP in the case of pWDH445) such that the signal from the reporter is significantly greater when DNA damage occurs. pWDH445 comprises a RAD54 regulatory element and the inventors have found that this regulatory element may be replaced by an RNR regulatory element to provide a recombinant vector according to the present invention.

Preferred vectors according to the first aspect of the invention comprise pWDH445 (yEGFP-444 in WO 98/44149) with disruption of the *kan*MX3 gene in which an RNR regulatory element replaces the RAD54 regulatory element.

The *kan*MX3 gene may be disrupted such that a mutation occurs by means of deletion, substitution or addition of nucleotides provided that genetecin resistance is impaired.

The RNR regulatory element of the recombinant DNA molecule activates

expression of the reporter protein when DNA damage occurs. Such regulatory elements ideally comprise a promoter sequence which induces RNA polymerase to bind to the DNA molecule and start transcribing the DNA encoding for the reporter protein. The regulatory element may also comprise other functional DNA sequences such as translation initiation sequences for ribosome binding or DNA sequences that bind transcription factors which promote gene expression following DNA damage. Regulatory elements may even code for proteins which act to dislodge inhibitors of transcription from the regulated gene and thereby increase transcription of that gene.

Preferred regulatory elements are DNA sequences that are associated in nature with the regulation of the expression of RNR DNA repair proteins. For instance, the regulatory elements from genes such as RNR1, RNR2 and RNR3 from yeast may be used to make recombinant DNA molecules according to the first aspect of the invention.

A preferred regulatory element comprises the promoter and 5' regulatory sequences of the RNR2 gene. The RNR2 gene may be found on chromosome X of Saccharomyces cerevisiae. A preferred regulatory element may be derived from between co-ordinates 387100 and 398299 associated with the RNR2 gene on chromosome X as identified in the Saccharomyces cerevisiae genome database. It is more preferably derived from between co-ordinates 387100 and 393299. The database may be accessed by the World Wide Web at many sites. For example at genome-www.stanford.edu.

The regulatory element of RNR3 is particularly preferred. The sequence of this element is well known and is also illustrated in Figure 18. The RNR3 promoter is particularly suitable because its induction is DNA damage specific; there is low level expression under normal condition; and massive induction incurs in response to damage.

The DNA sequences that encode a light emitting reporter protein may code for any light emitting protein, however it is preferred that the DNA sequences code for a protein that is fluorescent.

Preferred DNA sequences that encode a light emitting reporter protein code for Green Fluorescent Protein (GFP) and light emitting derivatives thereof. GFP is from the jelly fish Aquorea Victoria and is able to absorb blue light and re-emits an easily detectable green light and is thus suitable as a reporter protein. GFP may be advantageously used as a reporter protein because its measurement is simple and reagent free and the protein is non-toxic.

Derivatives of GFP include DNA sequences encoding for polypeptide analogues or polypeptide fragments of GFP which are able to emit light. Many of these derivatives absorb and re-emit light at wavelengths different to GFP found endogenously in Aquorea victoria. For instance, preferred DNA molecules according to the first aspect of the invention have a DNA sequence that encodes the S65T derivative of GFP (in which serine 65 of GFP is replaced by a threonine). S65T GFP has the advantage that it is brighter than wild-type GFP (when excited at its longest-wavelength peak) and shows only slow photobleaching. Furthermore S65T GFP produces a good quantum yield of fluorescence and matches the output of argon ion lasers used in fluorescence activated cell sorters. Cells according to the second aspect of the invention which contain DNA molecules coding S65T GFP may be used according to the method of the fourth aspect of the invention and are particularly useful when light emission is measured from cell extracts.

A most preferred DNA sequence encodes for a yeast enhanced GFP (yEGFP) such as the GFP derivative described by Cormack et al. (1997) (in Microbiology 143 p303-311). Such yEGFP has an amino acid sequence which is biased for usage in yeast. Thus yEGFP is particularly suitable for transforming cells according to the second aspect of the invention which are yeast. Furthermore we have found that light emitted from yEGFP in such yeast is even greater than that emitted by S65T derivatives.

Recombinant DNA molecules coding yEGFP are also useful because yEGFP is less heat sensitive than nascent GFP.

Most preferred recombinant DNA molecules comprise a RNR regulatory

element operatively linked to a DNA sequence that encodes a GFP or light emitting derivative thereof.

The recombinant vector according to the first aspect of the present invention may for example be a plasmid, cosmid or phage. Such recombinant vectors are of great utility when replicating the DNA molecule. Furthermore recombinant vectors are highly useful for transforming cells with the DNA molecule and may also promote expression of the reporter protein.

Recombinant vectors may be designed such that the vector will autonomously replicate in the cytosol of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. A suitable element is derived from the 2μ plasmid. Such replicating vectors can give rise to multiple copies of the DNA molecule in a transformant and are therefore useful when over-expression (and thereby increased light emission) of the reporter protein is required.

Preferably recombinant vectors may be formed from PFA vectors or derivatives thereof which are known to the art (see Wach et al. (1994) Yeast 10 p1793-1808).

Most preferred recombinant vector according to the first aspect of the invention are pGenRNR2 or pGenRNR3 depicted in figures 15 and 16. The sequence of such preferred vectors are given in Figures 17 and 18.

According to the second aspect of the invention the recombinant vector is incorporated within a cell. Such host cells may be prokaryotic or eukaryotic. Suitable host cells include bacteria, plant, yeasts, insect and mammalian cells. Preferred host cells are yeast cells such as *Saccharomyces cerevisiae*. Yeast are preferred because they can be easily manipulated like bacteria but are eukaryotic and therefore have DNA repair systems that are more closely related to humans than those of bacteria. Another benefit of using yeast cells as a host is that DNA repair systems are inducible in yeast unlike in humans where the repair systems are largely constitutive.

Preferred yeast cells include:

(i) Y485 in haploid form;

(ii) Y486 (also known as FF18984) in haploid form;

(iii) Y485/486 in diploid form;

(iv) FY73

(v) YLR030w.alpha.; and

(vi) Y300.

These strains may all be found in national yeast strain collections.

We have found that (i), (ii) and (iii) above are particularly useful strains for use according to the method of the invention.

Host cells used for expression of the protein encoded by the DNA molecule are ideally stably transformed, although the use of unstably transformed (transient) cells is not precluded.

Transformed cells according to the second aspect of the invention may be formed by following procedures described in the Example. The cell is ideally a yeast cell (for instance one of the strains described above). Such transformed cells may be used according to the method of the third aspect of the invention to assess whether or not agents induce or potentiate DNA damage. GFP expression is induced in response to DNA damage and the light emitted by GFP may be easily measured using a fluorimeter as an index of the DNA damage caused. For instance, the light emitted by GFP at 511 nm (after excitation between 475 and 495 nm--e.g. 488 nm) in response to DNA damage, may be evaluated either in a suspension of a defined number of whole cells or from a defined amount of material released from cells following breakage. Alternatively light emitted by GFP at 520 nm may be evaluated through a 535nm filter.

The method of the third aspect of the invention is particularly useful for detecting agents that induce DNA damage at low concentrations. The methods may be used to screen compounds, such as candidate medicaments, food additives or

cosmetics, to assess whether it is safe to expose a living organism, particularly people, to such compounds.

Alternatively the method of the third aspect of the invention may be employed to detect whether or not water supplies are contaminated by DNA damaging agents or agents that potentiate DNA damage. For instance, the methods may be used to monitor industrial effluents for the presence of pollutants that may lead to increased DNA damage in people or other organisms exposed to the pollution.

When the methods are used to detect whether or not water supplies are contaminated, the cells according to the second aspect of the invention are ideally unicellular organisms such as bacteria, algae, protoza and particularly yeast.

The expression of light emitting reporter protein may be monitored according to the method of the invention from cell extracts or from samples containing intact, whole cells.

There are several advantages associated with the use of whole cells. As there is no requirement to break open cells, the number of treatment steps is reduced. The production of extracts requires cell-harvesting, washing, breakage with glass beads and centrifugation to clear the extract. The reduction in treatment steps also reduces the risk of errors arising in handling and makes the method much faster. Furthermore cell density and light emission can be made simultaneously giving greater sensitivity. Therefore the method of the invention is preferably performed by growing cells transformed with a recombinant vector according to the first aspect of the invention (such as pGenRNR2), incubating the cells with the agent which putatively causes DNA damage for a predetermined time and monitoring the expression of the light emitting reporter protein directly from a sample of the cells.

When whole cells are used they are preferably contained in low fluorescence growth medium. This can obviate the need to wash the cells before measurements are made and therefore reduce the number of steps in the method further. For instance, preferred yeast according to the third aspect of the invention may be grown in F1

medium (described in Walmsley et al. (1983) Mol. Gen. Genet. 192 p361-365 and the Example).

According to a preferred embodiment of the method of the invention FF18984 cells may be transformed with pGenRNR2 or pGenRNR3 and grown in F1 medium. A putative DNA damaging agent (e.g. a food additive or potential medicament or an agent contained within a water sample or effluent sample) may then be added to the F1 medium containing the cells. The cells are then allowed to grow for a defined period of time after which a sample of the cells is removed and fluorescence measured therefrom. This measurement may be effected by estimating the cell concentration and fluorescence in the sample using nephelometry (light scattering). For example, cells can be illuminated at 600 nm and the scattered light (at 600 nm) estimated at 90 degrees to the incident beam. The light emitted by GFP can be measured by excitation at 475-495 nm (e.g. 487 nm) and fluorescent light emitted at 518 nm measured at 90 degrees to the incident beam. Both measurements may be made in a single cuvette. A normalised GFP light emission is calculated by dividing the GFP fluorescence value by the whole cell light scattering value (at 600 nm). This embodiment of the invention has the advantage that it may be easily carried out with a minimum of steps (i.e an incubation period followed by direct fluorescence measurement).

The method of the invention should ideally employ sensitive fluorimeters and reduce light scattering in order that light emission can be accurately measured from the reporter protein. We have found that sensitivity can be improved by using a 487 nm filter which is introduced between the sample chamber and the emission-detector of the fluorimeter. Such a filter further reduces the impact of light scattering and improves the sensitivity of the method when samples containing whole cells are used.

Preferred methods of fluorescence detection and quantitation are given in 1.2.5.5 of the Examples.

A preferred method of fluorescence detection is described in US 6, 509, 161. This method is particularly useful when the light emitting report is GFP or a derivative thereof.

A preferred method of testing for DNA damage according to the third aspect of the invention comprises the steps of : (1) preparing a microplate for use in an assay; (2) conducting the assay in the microplates; (3) collecting and analysing the data; and (4) making a judgment on DNA damage and the consequences.

Details of steps (1) – (4) for conducting a most preferred test for DNA damage are given below.

(1) Microplate preparation.

Assays were carried out in 96 well, black, clear-bottomed microplates. For example Matrix ScreenMates, Cat. No. 4929, Apogent Discoveries, USA, or Corning (BV, Netherlands: Cat. No. 3651). A number of alternative microplates were assessed, though the variable background absorbance and fluorescence both within and between plates from individual manufacturers were generally unacceptable, leading to the currently preferred choice. It will therefore be appreciated that microplates used according to the invention preferably have consistent absorbance and fluorescence between plates and batches thereof.

The assay plates can be filled using a liquid handling robot. For example the MicroLabS single probe, from Hamilton GB Ltd., Birmingham or a Genesis 8-probe robot (Tecan UK Ltd. Theale. UK). Microplates can also be filled rapidly and effectively using a multi-channel pipette.

(2) Assay

The following standard protocol may be followed. A 1 mM stock of a test chemical, or sample containing an agent that putatively caused DNA damage, was prepared in 2% v/v aqueous DMSO, and used to make 2 identical dilution series across a 96 well microplate - a test series and a control series. To achieve this, 150 microlitres of the test chemical solution were put into 2 microplate wells. Each sample was serially diluted by transferring 75 microlitres into 75 microlitres of 4% DMSO, mixing, and then taking 75 microlitres out and into the next well. This produced 9 serial dilutions of 75 microlitres each.

Controls were added as follows:

a. Test compound/ sample containing agent alone, to provide information on compound absorbance/fluorescence

b. Yeast cultures diluted with 4% DMSO alone, to give a measure of maximum proliferative potential

c. MMS as a genotoxicity control: 'high' = 0.00125% v/v, 'low' = 0.0001875% v/v

d. Methanol as a cytotoxicity control: 'high' = 3.5% v/v, 'low' = 1.5% v/v.

e. Growth medium alone, to confirm sterility/lack of contamination

Stationary phase cultures of yeast cells according to the invention. GenT01 were diluted to an optical density (OD600nm) = 0.2 in double strength F1 medium. 75 microlitres of the yeast suspension were added to each well of the diluted chemical. After the plates were filled, they were sealed using either a gas permeable membrane (for example Breath-easy, Diversified Biotech, USA) or a plastic lid, and then incubated without shaking, overnight at 25°C.

(3) Data collection and handling.

Following overnight incubation, fluorescence and absorbance data were collected from the microplates. Two different microplate readers which combine fluorescence and absorbance functionality have been used, and comparable data were obtained. These were a Tecan Ultra-384 (Tecan UK Ltd.): excitation 485 nm / emission 535 nm with an additional dichroic mirror (reflectance 320 nm – 500 nm, transmission 520 nm – 800 nm), and a BMG PolarStar (BMG Labtechnologies, Germany): excitation 485 nm / emission 520 nm. Absorbance was measured through a 620 nm filter in both instruments. The data were transported into a Microsoft Excel spreadsheet, and converted to graphical data. Data processing is minimal: absorbance data give an indication of reduction in proliferative potential and these data were normalised to the untreated control (=100% growth). Fluorescence data were divided by absorbance data to give 'brightness units', the measure of average GFP induction per cell. These data were normalised to the untreated control (=1). In this way, one can distinguish between a small number of highly fluorescent cells and a large number of weakly fluorescent cells. In order to correct for induced cellular autofluorescence and intrinsic compound fluorescence, the brightness values for a

control strain were subtracted from those of a test strain. This makes visual assessment of the data more reliable. All the data were checked with and without this correction, and the decision (see below), on whether or not a compound was classified as being genotoxic, was not affected.

(4) Decision thresholds.

It is useful to have clear definitions of positive and negative results from routine assays and such definitions have been derived, taking into account the maximum noise in the system and data from chemicals where there is a clear consensus on genotoxicity and mechanism of action. Naturally it is also possible for users to inspect the numerical and graphical data and draw their own conclusions. For example an upward trend in genotoxicity data that did not cross the threshold might still distinguish two compounds. The decision thresholds were set as follows:

The cytotoxicity threshold is set at 80 % of the cell density reached by the untreated control cells. This is greater than 3 times the standard deviation of the background. A positive cytotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a final cell density lower than the 80% threshold. A strong positive cytotoxicity result positive (++) is concluded when either (i) three or more compound dilutions produce a final cell density lower than the 80% threshold or (ii) at least one compound dilution produces a final cell density lower than a 50% threshold. A negative result (-) is concluded when no compound dilutions produce a final cell density lower than the 80% threshold. The lowest effective concentration (LEC) is the lowest test compound concentration that produces a final cell density below the 80% threshold.

The compound absorbance control allows a warning to be generated if a test compound is significantly absorbing. If the ratio of the absorbance of the compound control well to a well filled with diluent alone is > 2, there is a risk of interference with interpretation. The cytotoxicity controls indicate that the yeast is behaving normally. The 'high' methanol standard should reduce the final cell density to below the 80% threshold, and should be a lower value than the 'low' standard.

The genotoxic threshold is set at a relative GFP induction of 1.3 (i.e. a 30% increase). This is greater than 3 times the standard deviation of the background. A positive genotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a relative GFP induction greater than the 1.3 threshold. A strong positive genotoxicity result (++) is concluded if either (i) three or more compound dilutions produce a relative GFP induction greater than the 1.3 threshold or (ii) at least one compound dilution produces a relative GFP induction greater than a 1.6 threshold. A negative genotoxicity result (-) is concluded where no compound dilutions produce a relative GFP induction greater than the 1.3 threshold. The LEC is the lowest test compound concentration that produces a relative GFP induction greater than the 1.3 threshold. The genotoxic controls demonstrate that the strains are responding normally to DNA damage. The 'high' MMS standard must produce a fluorescence induction > 2, and be a greater value than the 'low' MMS standard. Anomalous brightness data is generated when the toxicity leads to a final cell density less than 30% that of the blank. Genotoxicity data is not calculated above this toxicity threshold. Compounds that tested negative for genotoxicity, were re-tested up to 10mM, or to the limit of solubility or cytotoxicity.

The compound fluorescence control allows a warning to be generated when a compound is highly auto-fluorescent. If the ratio of the fluorescence of the compound control well to a diluent filled well is >5, there is a risk of interference with interpretation. In these cases (four in this study), fluorescence polarisation can be used to distinguish GFP from other sources of fluorescence Both the Tecan and BMG instruments have this facility. Occasionally, compounds though not fluorescent themselves, induce cellular auto-fluorescence. This is apparent from rising brightness in the control strain in the absence of fluorescence from the chemical-only control. The routine subtraction of control from Test data removes this interference from the data.

The present invention will now be described, by way of examples, with reference to the accompanying drawings in which:

Figure 1 shows the difference between the brightness values obtained from FF18984 cells transformed with pWDH445, exhibiting normal levels of brightness (■) and

enhanced brightness (■) without and with exposure to MMS. U, untransformed FF18984 cells not treated with MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, cells transformed with pWDH445 but not treated with MMS; T MMS, cells transformed with pWDH445 and exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of three independent cultures. Both uninduced and MMS-induced brightness values are clearly significantly enhanced in the new transformants relative to the original transformants.

Figure 2 shows an ethidium bromide stained agarose gel of pWDH445 cut with *Bam*H I and *Asc* I. The lanes contain the following: Lane 1, pWDH445; Lane 2, plasmid isolated from an enhanced brightness transformant; Lane 3, pWDH445 isolated from a normal brightness yeast transformant; and Lane 4, a second isolate from a transformant exhibiting enhanced brightness. *Bam*H I-*Asc* I restriction of pWDH445 liberates a fragment of 3.2 kb (boxed) bearing *HO-RAD54*promoter-*yEGFP* cassette. A band representing a 3.2 kb-sized fragment is visible in all four lanes.

Figure 3 shows FF18984 cells transformed with pWDH445 and cells re-transformed with re-isolated pWDH445 or rearranged plasmid (two independent isolates) isolated from transformants of enhanced brightness. The four sets of transformants were replica-plated onto SD medium deficient in uracil and YPD medium incorporating 200 μgml^{-1} geneticin (G418) and incubated at 30°C for 3 days. All four sets of transformants grew on SD-ura but only the cells bearing pWDH445 and re-isolated pWDH445 grew on YPD+G418.

Figure 4 demonstrates that FF18984 cells re-transformed with rearranged plasmid (■) are brighter than FF18984 cells bearing pWDH445 (■) without and with exposure to 0.005% MMS. U, untransformed FF18984 cells not exposed to MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, cells transformed with pWDH445 or rearranged plasmid not exposed to MMS; T MMS, cells transformed with pWDH445 or rearranged plasmid and exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of six independent cultures. Cells re-transformed with the re-isolated rearranged plasmid are still brighter than normal pWDH445 transformants both without and with exposure to MMS.

Figure 5 shows the restriction map of pWDH445 created for and used in this study. Base-pair sequences of the plasmid constituents were analysed for restriction enzyme cleavage sites using software available on the Stanford Genome Database (SGD) web-site (see internet references). The map shows all of the restriction cleavage sites utilised (tested) in this chapter labelled with lines and appropriate enzyme names (italic-style font was not available in the drawing package used). Major plasmid components are shown as blocked sections with the plasmid backbone (mainly bacterial sequences, such as the origin of replication) drawn represented by a line (–). The *RAD54*promoter (■) -*yEGFP* (■) cassette and the *kan*MX3 cassette (■) are shaded to highlight the main regions of interest, whilst Amp^r and *URA3* are represented by open

block arrows. The arrows represent the direction of transcription for individual components.

Figure 6 shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) digested with *Xba* I. The lanes were loaded as follows: Lane 1, pWDH445; Lane 2, rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, second rearranged plasmid; Lane 5, 500 bp ladder (5-0.5 kb) marker DNA. *Xba* I restriction of both rearranged plasmids produced a novel band representing a fragment of ~4.4 kb, which was not detected for pWDH445 and re-isolated pWDH445. The rearranged plasmids also appear to be missing one band of a 6 kb doublet observed for the pWDH445 digestions. Extra bands in lane 2 reveal that one rearranged plasmid is larger than pWDH445, while the loss of a band from the doublet (and no extra bands) suggest that the rearranged plasmid loaded in lane 4 is smaller than pWDH445.

Figure 7 shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) restriction digested with *Sca* I. The lanes were loaded as follows: Lane 1, 500 bp ladder (5-0.5 kb) molecular weight marker DNA; Lane 2, pWDH445; Lane 3, larger rearranged plasmid; Lane 4, re-isolated pWDH445; Lane 5, smaller rearranged plasmid. Bands representing fragments sized ~1.8 kb and ~4.1 kb are present in all four lanes (lanes 2-5) but the band representing a fragment of 1.3 kb (present for pWDH445 and re-isolated pWDH445) is not exhibited in the digestion of either rearranged plasmid (lanes 3 and 5).

Figure 8 presents an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) restriction digested with *Pst* I. Lanes were loaded as follows: Lane 1, 500 bp ladder molecular weight marker DNA; Lane 2, pWDH445; Lane 3, larger rearranged plasmid; Lane 4, re-isolated pWDH445; Lane 5, smaller rearranged plasmid. Lanes 2 and 4 produced identical banding patterns whilst those in lanes 3 and 5 (rearranged plasmids) are significantly different. Only lanes 2 and 4 exhibit a band representing a fragment of ~2.5 kb. Lanes 2 – 5 all exhibit bands representing fragments of ~1.6, ~1.4, and ~1.3 kb, but lanes 3 and 5 also showed an extra band representing a fragment of 1.5 kb.

Figure 9 shows an ethidium bromide stained agarose gel of *Pvu* I-digested pWDH445, re-isolated pWDH445, and rearranged plasmids. Lanes loaded as follows: Lane 1, pWDH445; Lane 2, larger rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, smaller rearranged plasmid; Lane 5, 500 bp ladder molecular weight marker DNA. Lanes 1 and 3 exhibited the expected banding pattern, whereas neither rearranged (lanes 2 and 4) plasmid exhibits bands representing fragments of 1.2 and 0.7 kb. The larger rearranged plasmid (lane 2) also shows two novel bands representing fragments of ~3.5 kb and 1 kb.

Figure 10 shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) digested with *Sac* I. Lanes were loaded with the following digested plasmids: Lane 1, pWDH445; Lane 2, larger rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, smaller rearranged plasmid; Lane 5, 500 bp ladder molecular weight marker DNA. *Sac* I liberates a 2.1 kb *kan*MX internal fragment from pWDH445 and both lanes 1 and 3 exhibit a band

representing a 2.1 kb fragment. Neither rearranged plasmid (lanes 2 and 4) reveals such a band after *Sac* I restriction.

Figure 11 presents an ethidium bromide stained agarose gel of *Xba* I-digested pWDH445 and pWDH445 with *kan*MX enzymically removed. Lanes were loaded as follows: Lanes 1 and 13, pWDH445; Lanes 2-6, 8, 9, 11, and 12, plasmid isolated from ampicillin-resistant, kanamycin-sensitive *E. coli* transformants; Lane 10, plasmid isolated from an ampicillin-resistant, kanamycin-resistant *E.coli* transformant; Lane 7, 500 bp ladder molecular weight marker DNA. Lanes 1 and 13 exhibit a doublet band representing two fragments of ~6 kb, which is also seen for the plasmid from the kanamycin-resistant colony (lane 10). All other lanes containing digested plasmid show a single band representing a fragment of ~6 kb and a second band representing a fragment of ~4.5 kb.

Figure 12 represents the brightness values of FF18984 cells transformed with modified pWDH445 (enzymically removed *kan*MX) (■) compared with those from cells transformed with pWDH445 (■). U, untransformed FF18984 cells not exposed to MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, FF18984 cells transformed with pWDH445 or modified pWDH445 but not exposed to MMS; T MMS, cells transformed with pWDH445 or modified pWDH445 exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of ten independent cultures. Cells bearing the modified pWDH445 exhibit enhanced brightness comparable to that of cells re-transformed with isolated rearranged plasmid.

Figure 13 shows a schematic of the flow-through fluorescence detector with a blown-up schematic of the detector orientations within the light-tight box, and a further blown-up cartoon of the excitation beam and emissions, relative to the flow cell. The top section shows the overall layout of the instrument, beginning with cultures incubating in a water-bath shaker. Alternatively, samples can be injected *via* the sample injection valve. Culture/sample is pumped peristaltically into the light-tight instrument enclosure (grey box), wherein excitation occurs and emission is detected. The source of the excitation beam is an argon-ion laser (■). Within the instrument enclosure (see panel blown-up from the grey box) is a duplicated detector set-up. A beam-splitting cube send excitation light (488 nm) to both sets of detectors, each of which consists of an optical flow cell, photomultiplier tube (PMT), silicon photodiode (SPD), and optical filters (see key panel for colour-coding). Fluorescence is detected by the PMT whilst scattered light (as a measure of cell or particulate density) is detected by the SPD, both of which are situated perpendicular to the direction of the excitation beam. Orientation of the excitation beam and the emission detection is simplified in the bottom panel.

Figure 14 depicts the detection of fluorescence polarisation with the flow-through fluorescence detector. Sample circulating through the flow cell is excited by plane-polarised laser light (vertical arrows represent the plane of polarisation) and scattered light is detected by an SPD placed at the other side of the flow cell in line with the beam of excitation. Emitted fluorescence is less polarised than the excitation light and this is represented by vertical, horizontal, and diagonal arrows around the emission, before reaching the polaroid filters. The two polaroid filters either side of

the flow cell are positioned in opposite orientations, such that one filter allows transmission of the vertical component of the fluorescence (**Parallel** orientation with respect to the plane polarised excitation light), whilst the other permits transmission of the horizontal component (**Perpendicular** orientation with respect to the plane of polarisation of the absorbed light). Fluorescence transmitted by the polaroid filter is detected by two PMTs (**PMT1** and **PMT2**).

Figure 15 shows the restriction map of pGenRNR2 a preferred recombinant vector according to the present invention.

Figure 16 shows the restriction map of pGenRNR3 a preferred recombinant vector according to the present invention.

Figure 17 shows full sequence of pGenRNR2 in GeneBank format

Figure 18 shows full sequence of pGenRNR3in GeneBank format

Figure 19 shows results for (A) a cuvette assay and; (B) a microplate assay for a test strain transfected with pGenRNR2 and using MMS as a test compound in Example 2

Figure 20 shows results for (A) a cuvette assay and; (B) a microplate assay for a test strain transfected with pGenRNR3 and using MMS as a test compound in Example 2

Figure 21 illustrates the Greenrack loading sequence according to Example 3.

Figure 22 illustrates a microplate layout according to Example 3.

EXAMPLE 1

The discovery of brighter cells carrying a mutant pWDH445 will be described. This brighter strain was compared with a typical pWDH445-bearing strain followed by phenotypic analysis of the new strain and molecular biological examination of the plasmid borne by these cells. The creation of brighter strains by basic genetic engineering is disclosed. Finally the expression vector was manipulated to replace the RAD54 regulatory element with RNR regulatory elements to produce recombinant vectors according to the invention.

1.2 MATERIALS & METHODS

1.2.1 Strains and Plasmids

1.2.1.1 Strains

Saccharomyces cerevisiae and *Escherichia coli* strains used in the Example are listed in **Table 1**, along with their respective genotypes.

Table 1

Strain	Organism	Genotype	Source
FF18984	*S. cerevisiae*	*MATa leu2-3, 112 lys2-1 his7-1 ura3-52*	F. Fabre
DH5α	*E. coli*	*SupE44* Δ*lac*U169 (ϕ80*lacZ*Δ15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Laboratory collection

1.2.1.2 Plasmids

pWDH445 – is illustrated in **Figure 5** and corresponds to yEGFP-444 as disclosed in WO 97/44149 (e.g. see Figure 12)

1.2.2 Media

1.2.2.1 Sterilisation

All media were prepared by dissolution of the components in distilled water before sterilisation by autoclaving for 20 minutes at 15 lbin^{-2} unless otherwise stated. Alternatively, sterilisation was performed by filtration through 0.22 μm diameter pore Millipore filters where autoclaving was inappropriate.

1.2.2.2 Yeast media

Bacto-agar was added to a final concentration (w/v) of 2% to each of the growth media (1.2.2.2.1 –1.2.2.2.4). Yeast extract, peptone, and agar were all obtained from Difco (Becton Dickinson, Sparks, MD 21152, USA).

1.2.2.2.1 YPD (yeast extract, peptone, and dextrose)

Table 2

Component	% w/v	Final concentration gL^{-1}
Bacto-yeast extract	1	10
Bacto-peptone	2	20
Dextrose (glucose)	2	20
*Bacto-agar	2	20)

*Bacto-agar added in preparation of solid medium.

YPD was used as a rich growth medium to obtain good yeast cell yield, for unselective growth, and for selection of cells carrying antibiotic resistance by addition of antibiotic to the medium at the appropriate concentration. Glucose was diluted from a 40% stock (w/v) to give a final concentration of 2%, but was only added as required since it caramelises when autoclaved in YPD. Bacto-agar was added (2% w/v) in order to prepare solid medium.

1.2.2.2.2 – YPG (synthetic dextrose)

Table 3 - YPG

Component	% w/v or v/v	Final concentration gL^{-1} or mlL^{-1}
Bacto-yeast extract	1	10
Bacto-peptone	2	20
Glycerol	3 (v/v)	30 ml
*Bacto-agar	2	20

*Bacto-agar added in preparation of solid medium.

YPG is a complex medium containing glycerol as a non-fermentable carbon source. ρ^- or "petite" mutants are respiratory deficient mutants that cannot use glycerol as a carbon source and thus cannot grow on YPG. Again the medium was autoclaved before addition of the carbon source and Bacto-agar was added to 2% w/v for solid medium.

1.2.2.2.3 - SD

Table 4 -SD medium

Component	% w/v	Final concentration gL^{-1}
**Bacto-yeast nitrogen base	0.17	1.7
$(NH_4)_2SO_4$	0.5	5
Dextrose (glucose)	2	20
*Bacto-agar	2	20

*Bacto-agar is added in the preparation of solid medium.

** Bacto-yeast nitrogen base was purchased without amino acids or ammonium sulphate. This allows control of the nitrogen source and facilitates the preparation of selective media. A 20x YNB stock comprising $34gL^{-1}$ Bacto-yeast nitrogen base and $100\ gL^{-1}$ $(NH_4)_2SO_4$ was stored in the dark at 4°C.

SD is a synthetic defined minimal medium used for selection of auxotrophic mutants by controlling the nutritional supplements provided (amino acids, nucleic acid bases etc.). Supplements primarily used in this work were adenine sulphate (Ade), L-histidine (His), L-leucine (Leu), L-lysine (Lys), L-tryptophan (Trp), and uracil (Ura). These were used as described (Sherman *et al.*, 1986) and as shown in **Table 2.2.5.** YNB was typically made as a 20x stock solution including ammonium sulphate, as above. In the preparation of SD, the YNB was diluted to its 1x working concentration. SD was supplemented with the appropriate amino acids and bases in the quantities shown below. YNB and all amino acids and bases were obtained from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO 63178, USA).

Table 5 – SD Supplements

Component	Working concentration mgL^{-1}	Stock solution g/100 ml	Volume of stock mlL^{-1}
Adenine sulphate	20	0.2	10
Uracil	20	0.2	10
L-histidine	20	1.0	2
L-leucine	30	1.0	3
L-lysine	30	1.0	3
L-tryptophan	20	1.0	2

1.2.2.2.4 – F1 medium (fermentation one)

Table 6 - F1 medium - salts stock (6.7x)

Component	[x6.7] gL^{-1}	Final conc. gL^{-1}
$(NH_4)_2SO_4$	20.88	3.13
KH_2PO_4	13.33	2.00
$MgSO_4 . 7H_2O$	3.66	0.55
$CaCl_2 . 2H_2O$	0.60	0.09
NaCl	0.67	0.10

Table 7 - F1 medium - trace elements stock (10000x)

Component	[x10000] gL^{-1}	Final conc. mgL^{-1}
$ZnSO_4 . 7H_2O$	0.7	0.07
$FeCl_3 . 6H_2O$	0.5	0.05
$CuSO_4 . 5H_2O$	0.1	0.01
H_3BO_3	0.1	0.01
KI	0.1	0.01

Table 8 - F1 medium – vitamins stock (600x)

Component	[x600] gL^{-1}	Final conc. mgL^{-1}
Inositol	37.2	62
Thiamine/HCl	8.4	14
Pyridoxine	2.4	4
Ca-pantothenate	2.4	4
Biotin	0.18	0.3

F1 medium is a defined minimal medium used in fermentation [Brown *et al*, 1981, adapted by Walmsley *et al.*, 1983] that has a particularly low background autofluorescence. Concentrated stocks of salts, trace elements and iron (III) chloride were autoclaved, stored separately, and diluted into sterile water upon requirement. A concentrated vitamin stock was prepared in sterile water, filter sterilised by syringe and sterile 0.20 μm pore filter (Sartorius, Gottingen, Germany), and stored in aliquots at -20°C. Vitamins were then added subsequent to autoclaving of F1, to avoid their denaturation. All F1 was stored at 4°C in the absence of glucose.

A subsequent variant form of F1 was employed with 50% less inositol than quoted in the vitamins stock **Table 8** in order to lessen the effects of flocculation in troublesome yeast strains. This reduced inositol F1 was further modified by producing it in phosphate buffer at pH6 (as described in Varley, fourth edition, 1967) instead of in water. The buffered medium was created by mixing 0.067 M solutions of KH_2PO_4 and Na_2HPO_4, or concentrated stocks thereof (see **Table 9**), before addition of salts, trace elements, iron chloride, and nutritional supplements. The buffer mix was autoclaved and allowed to cool down to room temperature before addition of vitamins.

Table 9 - Phosphate buffer for F1 medium

Component	Volume of 1/15 M PO_4^{2-} /100 ml ml
Na_2HPO_4	12.2
KH_2PO_4	87.8

1.2.2.3 Bacterial media

The only bacterium used was *E. coli* which was grown on 3 different media; LB, SOB, and SOC.

1.2.2.3.1 – LB medium

Table 10 - LB Medium

Component	Concentration gL^{-1}
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	10

Luria-Bertani medium (LB) for broth cultures was prepared as described [Sambrook *et al.*, second edition, 1989] and LB broth plus 2% Bacto-agar was used for solid cultures.

1.2.2.3.2 - SOB

Table 11 - SOB

Component	Final concentration gL^{-1}
Bacto-tryptone	20
Bacto-yeast extract	5
NaCl	0.5

SOB was prepared as described in Sambrook *et al.* (second edition, 1989). After autoclaving the above components in 950 ml of water, 10 ml of 250 mM KCl were added before shifting the pH to 7 with 5 N NaOH. Immediately prior to use, 5 ml of 2 M $MgCl_2$ were added.

1.2.2.3.3 - SOC

SOC is identical to SOB except for the inclusion of 20 mM glucose after autoclaving.

1.2.3 Antibiotics

1.2.3.1 - Ampicillin

Ampicillin was prepared in aqueous solution at a stock concentration of 10 $mgml^{-1}$ and used at working concentrations of up to 100 μgml^{-1}. Ampicillin stock solutions were stored in aliquots of 1 ml at -20°C.

1.2.3.2 - Geneticin (G418)

G418 disulphate salt was prepared in aqueous solution as a stock solution at the concentration of 20 $mgml^{-1}$ and used at a working concentration of 200 μg/ml. The G418 stock solution was stored at 4°C.

1.2.3.3 - Kanamycin

Kanamycin was also prepared in aqueous solution, at a stock concentration of 50 $mgml^{-1}$ and used at a working concentration of 50 μgml^{-1}. Kanamycin stock solutions were stored at –20°C.

1.2.4 Standard solutions & Buffers

These are listed in alphabetical order for ease of navigation and those chemicals deemed to be carcinogenic or potentially carcinogenic are marked with an asterisk (*).

"Crushing" buffer: 20 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA

EDTA: 0.5 M EDTA (ethylenediaminetetra-acetic acid) pH 8.0 (pH adjusted to 8.0 by the addition of sodium hydroxide pellets)

Ethidium bromide*: A stock solution at a concentration of 10 $mgml^{-1}$ was prepared in sterile distilled water and stored in the dark at 4°C. It was diluted to a working concentration of 0.5 μgml^{-1} as required.

"Extraction" buffer: 20 mM Tris-HCl pH 7.0; 0.1M NaCl; 1 mM EDTA

Lithium acetate: 1 M lithium acetate (This was a 10x concentrated stock solution for lithium acetate transformation).

10x Loading dye: 25% w/v Ficoll (type 400, Pharmacia); 0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol FF

Lysing solution: 0.25 M Tris-HCl pH 7.5; 25 mM EDTA; 2.5% SDS

Methylmethane sulphonate (MMS, methanesulphonic acid methyl ester)*: Liquid stock solution diluted as required. Fresh diluted stock solutions were prepared for each experiment; 5 µl 100% MMS and 995 µl sterile water for a 0.5% (v/v) stock solution; **NB** All MMS concentrations quoted throughout this thesis are v/v percentages.

Polyethylene glycol: 50% w/v PEG 4000

Phenylmethylsulphonyl fluoride (PMSF): 0.1 M PMSF; Stored in isopropanol at 4°C.

Potassium acetate: 5 M potassium acetate

Restriction endonuclease buffers: The buffers recommended by the manufacturers were used unless otherwise stated.

RNAase: 10 $mgml^{-1}$ RNAase A; Prepared in a similar way to the protocol described in Sambrook et al. (second edition, 1989). 10 $mgml^{-1}$ RNase A was dissolved in 0.01 M potassium acetate (pH 5.2) and incubated at 100°C for 15 minutes. It was allowed to cool slowly to room temperature before the pH was adjusted by the addition of 0.1 volumes of 1M Tris-HCl (pH 7.5).

S-buffer: 10 mM $NaHPO_4$ pH7.2; 10 mM EDTA; 50 mM 2-mercaptoethanol; 10 $mgml^{-1}$ Novozyme

Sodium azide: 2% w/v sodium azide (NaN_3) stock solution

Sodium chloride: 1 M NaCl

Sodium dodecyl sulphate (SDS): 10% w/v SDS; The stock solution did not require sterilisation.

Sodium hydroxide: 10 N stock solution; Diluted as required and did not require sterilisation.

Solution I: 50 mM glucose; 25 mM Tris-HCl (pH 7.5); 10 mM EDTA (pH 8.0);

Solution II: 0.2 N NaOH; 1% SDS; Freshly prepared from concentrated stock solutions as required.

Solution III: 60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml distilled water

TB: 1.62 g PIPES; 12.5 ml 1 M KCl; 7.5 ml 1 M $CaCl_2$; 450 ml water; The pH was adjusted to 6.7 with KOH, made up to 495 ml with water, then 5.45 g Mn(II)-tetrahydrate added before filter sterilising through 0.45 µm pore filter unit.

5x TBE (Tris-borate, EDTA): 54g Tris base; 27.3g boric acid; 20 ml 0.5 M EDTA; Distilled water to make up to 1 L.

TE (Tris-EDTA): 100 mM Tris; 10 mM EDTA; 10x stock diluted to working concentration.
Tris-HCl pH 7.5: 1 M Tris; pH adjusted to 7.5 by drop-wise addition of HCl.

1.2.5 Experimental Procedures

1.2.5.1 - Growth Conditions

1.2.5.1.1 - *S. cerevisiae*

All yeast cultures grown on solid medium were statically incubated at 30°C in an Heraeus Electronic B5050 E temperature controlled incubator (Heraeus Instruments GmbH, Hanau, Germany). Liquid phase cultures were grown either in 250 ml conical flasks or 15 ml plastic centrifuge tubes. The temperatures used were 25°C or 30°C, and broth cultures were always shaken vigorously and continuously. Flask cultures were shaken in a Model G25 New Brunswick Scientific orbital incubator shaker (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). Centrifuge tubes were incubated either in a test tube rack fastened into the New Brunswick orbital shaker or in specially adapted Model G76 Gyrotory® Water Bath Shakers (New Brunswick Scientific Co. Inc.).

Solid phase cultures were maintained at 4°C as stock cultures for up to 6 months. These were used as sources of inocula for other agar plate cultures and liquid phase starter cultures. Stock cultures were also maintained at -80°C after growing 1.5 ml liquid cultures overnight and adding 1.5 ml of 30% glycerol (final 15% glycerol). The resultant 3 ml culture was split into 2 aliquots of 1.5 ml in sterile 2 ml screw-capped cryogenic vials (Nalgene, Nalge Co, Rochester, NY, USA).

1.2.5.1.2 - *E. coli*

Solid phase *E. coli* cultures were grown under temperature controlled conditions at 37°C in a temperature controlled incubator. Liquid cultures were grown with vigorous shaking at 37°C in a Model G25 New Brunswick Scientific orbital incubator shaker. Solid phase cultures were maintained at 4°C but were generally not re-used.

1.2.5.2 – Transformation protocols

1.2.5.2.1 – Lithium acetate transformation of yeast with plasmid DNA

Transformation of *S. cerevisiae* with plasmid DNA was performed by a rapid lithium acetate/PEG/single-stranded DNA method, adapted from that described by Gietz *et al.*, 1995. An initial "starter" culture of the appropriate yeast strain was prepared by scraping a loopful of frozen stock culture or by picking a portion of a colony with an inoculating loop from an agar plate stock culture, and inoculating 50 ml of YPD in a 250 ml conical flask. This starter culture was incubated overnight (~16 hours) at 30°C with shaking. A 10 µl aliquot was removed and diluted to 1 ml in a 1 ml plastic cuvette. A second cuvette was filled with 10 µl of fresh YPD medium duiluted to 1 ml, to act as a blank for optical density (OD) measurement. Using a Cecil Instruments CE505 Double Beam Ultraviolet Spectrophotometer with the wavelength set to 600 nm, the absorbance of the blank was set to zero before reading the OD of the diluted culture. Using the known OD of the starter culture, the culture was then sub-cultured to the required starting OD of approximately 0.2 in 50 ml of fresh YPD in a 250 ml conical flask. The sub-culture was incubated for 3 to 4 hours to allow approximately 2 complete generation times, to ensure that a large proportion of the culture is in the early to mid- exponential phase of growth required for transformation.

30 ml of the sub-culture were transferred to 50 ml Falcon tubes, and the cells harvested by centrifugation in an Eppendorf Centrifuge 5403 (Eppendorf, Hamburg, Germany) at 4000 rpm for 4 minutes. The supernatant was discarded and the cell pellet was washed (resuspended and then harvested again, followed by removal of the supernatant) twice in 10 ml of sterile water, harvesting by centrifugation as before. The resulting cell pellet was this time resuspended in 1 ml of sterile water and transferred to a sterile 1.5 ml microfuge tube. The cells were harvested in the microfuge tube by centrifugation in an Eppendorf Centrifuge 5414 (microfuge) for 30 seconds. The supernatant was discarded and the pellet washed in 1 ml of 0.1 M lithium acetate/1x TE (1x TE is 10 mM Tris-Cl pH7.5, 1 mM EDTA pH8.0), the supernatant removed and the pellet resuspended in 0.5 ml of 0.1 M lithium acetate/1x TE.

Sheared salmon sperm DNA, prepared by sonication, was boiled for 10 minutes in a boiling water bath to melt the double-stranded DNA into single-stranded "carrier" DNA. After boiling, the carrier DNA was immediately transferred to ice for 2 minutes. As long as single-stranded carrier DNA was immediately moved onto ice incubation and was re-frozen shortly after boiling, the salmon sperm DNA was only boiled every 4 or 5 times it was defrosted.

50 µl aliquots of cells in 0.1 M lithium acetate/1x TE were transferred to sterile microfuge tubes. To each aliquot were added ~1 µg of plasmid DNA, ~20 µg of single-stranded carrier DNA, and 300 µl of 40% PEG/1x TE/0.1 M lithium acetate. The contents of each microfuge tube were mixed carefully and thoroughly by pipette, before transferral of the tubes to a shaking incubator for 30 minutes incubation at 30°C with shaking. After 30 minutes at 30°C, the tubes were transferred to a water bath with the temperature set to 42°C. This heat shock incubation lasted for 15 minutes, although this could be varied depending on strain tolerance of such stress.

After the heat shock treatment, each transformation mix was further diluted with 0.5 ml of sterile water before harvesting the cells and removal of all traces of the PEG containing supernatant. To ensure that all PEG was removed, the cells were further washed carefully with 1x TE before eventually resuspending the cell pellets in 1 ml of fresh YPD by pipette. Cells were given 2 hours at 30°C with shaking, or ~16 hours overnight at 4°C to recover in YPD.

For selection of antibiotic resistant transformants, 150 µl of the 1 ml YPD culture of transformed cells were pipetted onto YPD-agar incorporating the selection antibiotic. Cells were spread onto the agar using an ethanol-flamed glass spreader under aseptic conditions and the agar plates allowed to dry in a laminar flow cabinet, before incubating at 30°C for 3 to 5 days.

If the method of transformant selection was suppression of auxotrophy, then the cells from the 1 ml YPD culture were harvested and the YPD supernatant removed. All traces of YPD had to be removed since even small amounts can permit background growth of non-transformants. Hence, after removal of the YPD supernatant, the cell

pellets were washed with 1x TE before finally resuspending in 1 ml 1x TE. 150 µl of each 1x TE suspension were transferred to SD-agar plates incorporating the required nutritional supplements except one for which the untransformed cells were auxotrophic, to allow for selection of cells that have become prototrophic for the missing nutrient through transformation. Again the cell suspensions were spread using a glass spreader with asceptic technique and allowed to dry in a laminar flow cabinet before incubation at 30°C for 3 to 5 days.

All transformants (auxotrophic or antibiotic selected) were replica plated onto sets of SD-agar plates, each with one required nutrient missing. This was performed in order to check that transformants had the correct marker gene phenotype. True transformants should grow on plates containing all the nutrients required by untransformed cells, or on plates lacking only the nutrient for which transformants acquire prototrophy (due to transformation).

1.2.5.2.2 - Transformation of *E. coli* with plasmid DNA

1.2.5.2.2.1 - Preparation of competent *E. coli* cells

Competent *E. coli* cells were prepared by the following protocol (J. Schmuckli-Maurer, Institute of Cell Biology, University of Bern, personal communication). A starter culture of *E. coli* cells (DH5α strain) was prepared in a volume of typically ~5 ml of LB broth in a universal tube. This culture was incubated overnight at 37°C with shaking and then sub-cultured into a 2 L flask containing 250 ml of SOB medium, to give an initial OD_{600nm} of approximately 0.1. The sub-culture was incubated with shaking at room temperature for 18 to 24 hours before being incubated on ice for 10 minutes. After ice incubation the cells were harvested by centrifugation at 8000 rpm in an Eppendorf Centrifuge 5403. The supernatant was discarded and the pellet resuspended in 80 ml of TB buffer and the tubes incubated on ice for another 10 minutes. Again the cells were harvested and the TB supernatant discarded and this time the pellets resuspended in 20 ml of TB buffer. To the 20 ml TB buffer cell suspension, 1.4 ml of DMSO were added and mixed gently before another 10 minutes ice incubation. 1 ml aliquots of the competent cells in 1.5 ml microfuge tubes were snap-frozen in liquid nitrogen and stored in a -80°C freezer.

1.2.5.2.2.2 - Transformation of competent *E. coli* cells with plasmid DNA

The following *E. coli* transformation protocol is taken from Inoue and co-workers (1990). Aliquots of competent cells stored at -80°C were allowed to defrost at room temperature. 1.5 ml microfuge tubes were pre-cooled before the addition of ~100 ng of plasmid DNA. 200 µl of defrosted competent cells were added to the DNA and incubated on ice for 30 minutes. After 30 minutes, the *E. coli*/DNA mixtures were transferred to a 42°C water bath for 30 seconds for heat shock treatment. Immediately after the heat shock, the tubes were cooled by ice incubation. To each transformation tube, 0.8 ml of SOC medium (though LB with 2% glucose added suffices) were added and these suspensions were shaken for 1 hour at 37°C.

Transformed *E. coli* were antibiotic resistant, so the SOC or LB used for recovery of the cells at 37°C did not have to be removed to limit background growth. 100 to 150 µl of transformed cells were spread onto LB-agar incorporating the antibiotic required for selection, using aseptic technique. The agar plates were allowed to dry in a laminar flow cabinet and were then incubated at 37°C overnight.

1.2.5.3 – Isolation of plasmid DNA

1.2.5.3.1 - Preparation of plasmid DNA from yeast cells (yeast "mini-prep")

The protocol described here for the preparation of plasmid DNA from yeast cells was provided by J. Schmuckli-Maurer (University of Bern) in a personal communication. Yeast scraped from a plate was resuspended in 1 ml of sterile water in a microfuge tube. The OD_{600nm} of this suspension was adjusted to between 2 and 5 units. Alternatively, cells were taken from a small (1-5 ml) overnight culture. Cells were harvested by centrifugation and the supernatant discarded. Cell pellets were resuspended in 0.5 ml of S buffer and incubated at 37°C for 30 minutes. 100 µl of lysing solution were added to each cell suspension and mixed by vortexing and then incubated at 65°C for 30 minutes. 96 µl of 5 M potassium acetate (pH 5.2) were added to each tube before incubating on ice for 10 minutes. Cell debris was precipitated by centrifugation in a microfuge for 10 minutes and the resulting supernatants were removed to fresh sterile microfuge tubes. DNA was precipitated from the supernatants by the addition of 800 µl of cold ethanol, 10 minutes ice incubation and then centrifugation in a microfuge for 10 minutes. The ethanolic

supernatants were discarded and traces of ethanol removed by aspiration. Pellets were washed with 500 µl of 70% ethanol and then allowed to air-dry. Dry pellets were resuspended in 40 µl of sterile water and 5 µl (~100 ng) of this preparation were used to transform competent *E. coli* cells. Remaining DNA was stored at -20°C.

1.2.5.3.2 - Preparation of plasmid DNA from *E. coli* cells (bacterial "mini-preps")

Small-scale preparation of plasmid DNA from *E. coli* was performed by alkaline lysis as described in Sambrook *et al.* (second edition, 1989). Single bacterial colonies were transferred to individual sterile 15 ml test tubes containing 2 ml of LB broth medium plus the appropriate antibiotic for the maintenance of plasmid selection. The cultures were incubated overnight at 37°C with vigorous shaking. 1.5 ml aliquots were taken from each culture and transferred to 1.5 ml microfuge tubes. Cells were harvested by centrifugation in a microfuge for 30 seconds and then the supernatant removed by aspiration (using a disposable pipette tip attached to a vacuum line) to leave the pellet as dry as possible.

The bacterial pellets were resuspended in 100 µl of ice-cold solution I by vigorous mixing. 200 µl of freshly prepared solution II were added to each suspension and mixed together by rapid inversion of the tubes. The tubes were chilled on ice whilst 150 µl of solution III were added to each mixture. The contents of the tubes were mixed whilst inverted to ensure appropriate mixing before incubating on ice for 5 minutes. DNA was precipitated by addition of 2 volumes of ethanol at room temperature and vortexed to mix. This was allowed to stand for 2 minutes before harvesting the DNA by centrifugation in a microfuge for 5 minutes. The supernatants were removed by aspiration (tip and vacuum line, as before) and the pellets were allowed to air-dry, prior to rinsing the DNA pellets with ice-cold 70% ethanol. The supernatants were again aspirated off, taking care not to dislodge the pellets. Pellets were allowed to air-dry again before being resuspended in 50 µl of TE containing 20 $\mu g ml^{-1}$ DNase-free RNAase. DNA preparations were stored at -20°C.

1.2.5.4 – Manipulation of DNA

1.2.5.4.1 - Restriction enzyme cleavage of plasmid DNA

Enzymes and buffers were obtained from Boehringer Mannheim (now Roche Diagnostics Ltd., Lewes, UK) and digests were carried out according to the manufacturer's instructions. A typical restriction enzyme digest was carried out as follows for a 10 µl reaction: 1 µl sample DNA (approximately 25 to 75 ng) isolated from *E. coli* by method **1.2.5.3.2**, 1 µl of the appropriate enzyme buffer, 1 µl enzyme (~10 units), and sterile water to make the volume up to 10 µl. The reaction was incubated at 37°C for between 1 and 2 hours or until the DNA was digested.

1.2.5.4.2 - Ligation of "sticky-ended" DNA termini

Plasmid DNA was cut by digestion with restriction endonuclease, as described above, to leave single-stranded overhangs or "sticky ends". The success of the digestion can be checked by agarose gel electrophoresis of a small sample of restricted DNA. Digested DNA was incubated at 65°C for 20 minutes in order to inactivate most restriction enzymes. This DNA was then diluted to the appropriate concentration (~100 $\mu g ml^{-1}$) and chilled to 0°C. In order to re-join the ends of the plasmid without insertion of foreign DNA, the ligation reaction was set up as follows (as described in Sambrook *et al.*, 1989). Approximately 0.1 µg of DNA was transferred to a fresh sterile microfuge tube. To this were added 2 µl of 10x bacteriophage T4 DNA ligase buffer and 0.1 units of bacteriophage T4 DNA ligase. The reaction was made up to a final volume of 20 µl with the addition of sterile water. Reactions were typically incubated at 15 or 16°C (controlled temperature water bath in 4°C cold room) overnight. Bacteriophage T4 ligase and its respective buffer were from Boehringer Mannheim.

1.2.5.4.3 - Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using the standard procedure [Sambrook *et al.*, 1989]. 1% agarose gels were prepared with running buffer 0.5x TBE with 0.5 $\mu g ml^{-1}$ ethidium bromide. Lambda DNA digested with *Hind* III and *Eco* RI, and a 500 base pair (bp) ladder (500-5000 bp) were used as molecular weight markers. Both types of marker DNA, along with the restriction enzymes and buffers, were from Boehringer Mannheim.

1.2.5.5 – Fluorescence detection and quantitation

1.2.5.5.1 - Fluorescence assays with cell extracts

The assessment of GFP fluorescence in cell extracts was performed essentially as described in Walmsley *et al.*, 1997 (Yeast 13 p1535-1545). A stationary phase culture of cells grown in SD medium was used as the inoculum source. 15 µl aliquots of cells were inoculated into 1.5 ml of SD in 15 ml test tubes. Half of the tubes were then supplemented with 0.01% MMS (the remaining tubes left as controls). The tubes were incubated at 25°C (accurate folding of the S65T derivative of GFP is temperature sensitive) for 16 hours in an orbital shaker incubator at 120 rpm, or in a water bath shaker. After 16 hours the cultures were adjusted to 0.02% NaN_3 to inhibit respiration and incubated on ice for 90 minutes, maintaining agitation. The cells were then transferred to 1.5 ml microfuge tubes, harvested by centrifugation (10s), washed twice in sterile distilled water, then washed in 1 ml of "extraction" buffer (20 mM Tris-Cl pH 7.5, 0.1 M NaCl, and 1 mM EDTA). After harvesting the cells by centrifugation, the supernatant was aspirated off and the cell pellet resuspended in 250 µl "crushing" buffer (20 mM Tris-HCL, pH 7.5, 0.1 M NaCl, and 1 mM PMSF). 250 µl of 400-600 nm diameter glass beads were added to the tubes of cells that were then placed in a BIO101 Fastprep FP120 (Savant, purchased from Anachem Ltd, Luton, UK) on speed 4 for 30 seconds to mechanically disrupt the cells. The tubes were incubated on ice for 1 minute and then returned to the Fastprep for another 30 seconds. This process was repeated such that each tube had undergone 3 periods in the Fastprep separated by 1 minute ice incubations. Following centrifugation for 30s, the supernatant was transferred to a clean tube. The pellet of beads was washed in a further 250 µl of crushing buffer and this was added to the supernatant from the previous extraction. The extract was adjusted to 0.1 M Tris, pH 11 by the addition of 1 M Tris base, 200 µl of the pooled extract plus 2.8 ml of water was then transferred to an acrylic cuvette (Sarstedt Ltd, Numbrecht, Germany).

1.2.5.5.2 - Fluorescence assays with intact yeast cells

Starter cultures were initiated by inoculating 1.5 ml of F1 medium (plus appropriate nutritional supplements and 2% glucose) in sterile 15 ml polythene centrifuge tubes with small portions of colonies, picked by inoculating loop from stock plates. These

starter cultures were grown for up to 24 hours at 30°C with shaking (at 120 rpm) and were used as the source of inoculum for the assay cultures. Cells were inoculated into 1.5 ml F1 cultures in 15 ml polythene centrifuge tubes to give an initial OD_{600nm} of ~0.1 (typically a 10-15 µl inoculum per 1.5 ml culture). The lids of the tubes were left one-quarter unscrewed, but held in place by masking tape, to ensure maximum oxygenation for GFP maturation. Half of the tubes were treated with 0.005% methyl methanesulphonate (MMS, methanesulphonic acid methyl ester) and half left as unchallenged controls. MMS was purchased as a liquid and this stock solution was taken to be 100%. This was diluted to a 0.5% stock in small aliquots as required and then diluted 1:100 for the assay cultures (15 µl of 0.5% stock per 1.5 ml culture). Assay cultures were incubated at 25°C (30°C when the S65T-GFP was replaced by yEGFP, which is more heat stable) in shaking water bath incubators for 14-16 hours. Each of the 1.5 ml cultures was transferred to a 4-window acrylic cuvette (Sarstedt Ltd, Numbrecht, Germany) and diluted with 1 ml of sterile water before measurement. For the measurement of intact yeast cell fluorescence of cells grown in YPD or SD, the cells were washed twice in sterile water to remove traces of autofluorescent medium and resuspended in 1.5 ml of sterile water. Washed cells were then transferred directly to 4-window acrylic cuvettes containing 1 ml of sterile distilled water. 4-window cuvettes were necessary since measurement of fluorescence emission is performed perpendicular to the path of excitation light.

1.2.5.5.3 - Fluorescence measurements and "Brightness" calculation

Fluorescence measurements were performed with a Perkin-Elmer LS50B Fluorescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, UK). The excitation and emission wavelengths were set to 488 nm and 511 nm respectively, with a slit width of 10 nm, for the S65T GFP derivative- expressing reporter. For the yEGFP expressing reporter, the excitation and emission wavelengths were set to 490 nm and 518 nm respectively (due to the altered fluorescence characteristics of yEGFP), with 5 nm slit widths. To account for differences in cell numbers between YPD or SD assay cultures, the OD_{600nm} was recorded for each cuvette. Similarly, in order to measure protein extraction efficiency, light absorption at 280 nm (OD_{280nm}) was measured for crude cell extracts. Both of these measurements used a Cecil Instruments CE505 double beam ultraviolet spectrophotometer. Acrylic cuvettes were essential for the

latter measurements since they permit greater transmission of ultraviolet (UV) light. Cell density estimations for F1 medium cultures were made by measuring light scattering (nephelometry) using the LS50B luminescence spectrometer, with both excitation and emission wavelengths at 600 nm and slit widths of 2.5 nm (Neph600). The fluorescence values obtained from the fluorometer were then divided by the absorption/scatter readings to give the "brightness value", an arbitrary unit which is independent of sample concentration, though varies with different fluorometers. The y-axis on uncorrected fluorescence scans gives raw data in the form of machine defined fluorescence intensity ("INT") units.

1.2.5.5.4 – Induction ratio

The induction ratio is used to calculate a "signal-to-noise ratio" with respect to the GFP signal. All detected signals are not pure GFP signals, but incorporate contaminating background fluorescence signals (autofluorescence). Autofluorescence significantly varies with the changing growth phase of a culture (see Chapter 5) and is dependent on the strain background. Hence, in order to be able to compare the signal-to-noise ratios from cultures of the same strain in different growth phases or from cultures of different strains, it is necessary to remove the variable autofluorescence component from the brightness values.

Firstly, several terms (used throughout this thesis) must be defined:

"U" is the brightness value from untransformed cells *i.e.* cells not bearing a reporter plasmid (or other plasmid where specified), that have not been exposed to a genotoxin such as MMS, and as such this value represents the uninduced autofluorescence.

"U MMS" is the brightness value from untransformed cells that have been exposed to a genotoxin (in this case, MMS). This value represents the genotoxin-induced level of autofluorescence, often larger than that of uninduced autofluorescence.

"T" is the brightness value from cells transformed with one of the *RAD54*-GFP reporter plasmids (or other plasmid where specified) that have not been exposed to a genotoxin. This value represents the constitutive level of GFP fluorescence due to

continual low-level expression from the *RAD54* promoter, and also incorporates the uninduced autofluorescence.

"T MMS" is the brightness value obtained from cells transformed with a plasmid that have been exposed to a genotoxin (again, MMS in this case). T MMS represents a combination of the GFP signal due to damage-induced up-regulation of expression from the *RAD54* promoter, the constitutive GFP signal, and the damage-induced autofluorescence signal.

For a true signal-to-noise ratio based solely on the signal from GFP, the two parameters of interest are the GFP signal due to the damage-induced response of *RAD54* and the constitutive GFP response in the absence of damage. The constitutive signal is obtained by subtracting the uninduced autofluorescence from T (*i.e.* T – U) and the induced GFP signal is calculated by subtracting the induced autofluorescence from T MMS (*i.e.* T MMS – U MMS). (T – U) gives the constitutive signal or C value, whereas (T MMS – U MMS) provides the induced value or I value. The induction ratio is a simple ratio of I to C and can be expressed thus:

$$\text{Induction Ratio} = \frac{(\text{T MMS} - \text{U MMS})}{(\text{T} - \text{U})} \qquad \frac{(\text{GFP signal})}{(\text{GFP noise})}$$

1.2.5.5.5 - Flow-through fluorescence detector

The development of a continuous-flow detector for the measurement of GFP signal from a yeast culture has been described previously [Knight *et al.*, 1999, Measurement Science and Technology, 10: 211, 217 and adapted for the fluorescence polarisation technique [Knight *et al.*, 2000a, The Analyst, 125: 499-506. **Figure 13** shows a schematic diagram of the basic layout of the instrumentation developed with a fluorescence flow cell, through which the yeast culture or GFP extract is circulated by use of a Gilson Minipuls 3 peristaltic pump (purchased from Anachem Ltd, Luton, UK). The 488 nm excitation was provided by an air-cooled 40 mW argon ion laser (LG Laser Graphics GmbH, Dieberg, Germany), reduced to 5 mW by filtering. A photomultiplier tube (PMT) was used as the fluorescence detector and a silicon photodiode (SPD) as the scattered light detector for nephelometric measurement of

the cell density. The flow cell, PMT, SPD, and associated electronics were housed in a light-tight box (in duplicate in **Figure 13**). The PMT is positioned to one side of the flow cell such that fluorescence is detected at 90° to the path of the excitation light. The SPD is situated at the other side of the flow cell from the light source in the path of the excitation light, or to one side of the flow cell in the same way as the PMT, since scattered light can detected in either orientation. Data acquisition and manipulation was performed in real time on a personal computer *via* a 12-bit analogue to digital converter (ADC) and associated software (Pico Technology Ltd., Cambridge, UK).

1.2.5.5.6 - Fluorescence polarisation

Figure 14 shows the detection of fluorescence polarisation using the flow-through fluorescence detector. Polarised laser light (polarisation represented by vertical arrows) is used to excite the sample circulating through the flow cell. In this cartoon, fluorescence is detected at 90° on either side of the flow cell with a single polaroid filter between the flow cell and each PMT. Fluorescence from the sample in the flow cell is less polarised than the excitation light (represented by vertical, horizontal, and diagonal arrows). The polaroid filters to either side of the flow cell are arranged in opposite orientations, such that one filter allows the vertical component of the fluorescence to pass through (parallel orientation, with respect to the plane of polarisation of the incident light), while the other permits transmission of only the horizontal component of the fluorescence (perpendicular orientation, with respect to the plane of polarisation of the incident light).

The degree of fluorescence polarisation (P) measured in this work was defined as:

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ is the fluorescence intensity measured polarised parallel to the absorbed plane-polarised radiation, and $I_{\perp}$ is that perpendicular to the absorbed radiation. P is a dimensionless parameter and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. For the discrimination of GFP signal from

that of autofluorescence, calculation of the ($I_{\parallel}$ - $I_{\perp}$) term is sufficient, since this term tends to zero for molecules emitting relatively unpolarised fluorescence (for example, small molecules like fluorescein), whilst being relatively large for molecules emitting fluorescence that exhibits significant polarisation.

1.2.5.5.7 Construction of recombinant vectors according to the present invention

pGenRNR3 was constructed by PCR amplifying the RNR3 promoter from yeast genomic DNA up to and including the ATG start codon from the strain F486, as a BamHI and PacI fragment. This PCR product was cut with BamHI and PacI and dropped into pGen001 also cut with BamHI and PacI. The pGenRNR2 plasmid was prepared in the same manner.

1.3 RESULTS

1.3.1 Brighter FF18984 transformants bearing pWDH445

1.3.1.1 - Spontaneous generation of bright pWDH445-bearing FF18984 cells

After the production of new pWDH445-containing strains, transformants screened by marker selection were assayed for a change in brightness in response to MMS, to ensure the presence of functional pWDH445.

Sporadically, pWDH445 transformants (transformed using experimental procedure **1.2.5.2**) were identified which were significantly brighter in response to MMS than typical transformants. **Figure 1** shows the average normal brightness for 3 replicates of FF18984 cells carrying pWDH445 (darker bars) and the average brightness for 3 replicates of brighter FF18984 cells carrying pWDH445 (lighter bars). In this example, 'T' (transformed cells with no MMS) was 12 fold brighter in the new variant FF18984 cells than the typical signal level, and 'T MMS' was 9 fold brighter. The enhanced brightness in the variant cells actually led to a decrease in the induction ratio from 7.72 to 3.27. However, the ease of detection of the variant cells with enhanced signal output made them attractive for the development of ever-brighter reporters.

1.3.1.2 - There is a link between enhanced signal output and geneticin resistance

The bright variants discussed above were first subjected to phenotypic analysis to confirm that they were indeed FF18984 transformants. Strains were re-plated onto media excluding uracil to check for the presence of the pWDH445. This was the favoured method of transformant selection to minimise the use of antibiotics (in this case G418). Both normal and variant transformed cells grew on SD medium lacking uracil. Cells were replica-plated onto SD medium lacking one of leucine, lysine, or histidine, and once more both normal and variant strains responded accordingly: *i.e.* they were unable to grow. This suggested that both normal and variant cells had the correct genetic background for FF18984.

The cells bearing pWDH445 were then tested for resistance to G418, which is conferred by the *kan*MX module carried on pWDH445. Cells of typical brightness were G418 resistant and hence able to grow on G418 (200 $\mu g ml^{-1}$) containing YPD

plates. However, bright variant cells were unable to grow on such medium, despite the previous confirmation of the presence of pWDH445 by growth on uracil-deficient medium. This suggested that mutation or rearrangement of the reporter plasmid had occurred in the variant cells.

1.3.1.3 - The *RAD54*-GFP cassette has no gross rearrangements

In order to test the hypothesis that the plasmid had undergone a rearrangement or mutation, it was necessary to isolate pWDH445 from both normal cells and variant cells. Plasmid was prepared from yeast as described in experimental procedure **1.2.5.2.2** and the DNA pellet was dissolved in 40 µl of sterile water. 5 µl of each plasmid preparation was used to transform DH5α *E. coli* cells by the protocol described in method **1.2.5.2.2**. *E. coli* transformed with the plasmids prepared from yeast were selected by growth on LB agar containing 80 $\mu g ml^{-1}$ ampicillin. Transformant cultures and plasmid preparations were made as described in the methods and DNA pellets were resuspended in 50 µl of TE and RNAase (20 $\mu g ml^{-1}$).

A simple test for the integrity of the *RAD54*-GFP cassette in pWDH445 was to utilise the modular nature of the plasmid to liberate the cassette by restriction endonuclease digestion (see experimental procedure **1.2.5.4.1**). This module is released by double digestion of pWDH445 with *Bam*H I and *Asc* I restriction enzymes to give a 3177 bp fragment from the normal plasmid. pWDH445, plasmid prepared from 2 independent bright transformants, and plasmid isolated from a normal transformant were digested with *Bam*H I and *Asc* I, and the resulting DNA fragments separated by agarose gel electrophoresis. **Figure 2** shows the bands produced after ethidium bromide staining for the above restriction digests. All 4 pWDH445 reactions produce a band of ~3.2 kb suggesting that the *HO-RAD54*promoter-*yEGFP* cassette is unchanged from the normal pWDH445 (lanes 1 and 3) to those isolated from bright variants (lanes 2 and 4). However, the band representing the remainder of the plasmid is larger in one of the variants (lane 2) and smaller in the other (lane 4), whilst it remains constant between the DNA used in transformation and that isolated from a transformant showing normal brightness levels.

1.3.1.4 - Yeast re-transformed with rearranged plasmid are G418 sensitive

Plasmid DNA isolated from yeast and then prepared from *E. coli* after amplification was used to re-transform FF18984 yeast with normal and rearranged pWDH445, using the lithium acetate / PEG / SS-DNA protocol described in method **1.2.5.2**. Transformants were selected by their ability to grow on medium deficient in uracil, since this was apparently unaffected by the mutations / rearrangements in the variants. The auxotrophic markers of transformants were checked as before and corresponded to those of the FF18984 background, with the exception of the ability to grow without uracil, conferred by pWDH445. Once more, resistance to G418 was tested by transferal of transformants to YPD plates containing 200 μgml^{-1} G418. **Figure 3** shows photographs taken of re-transformed FF18984 incubated on YPD plates containing G418 and SD plates lacking uracil. The uracil-deficient plates are represented in the right-hand column and as expected demonstrate growth in all cases. However, the G418 plates (left-hand column) reveal that cells carrying the control pWDH445 and the normal pWDH445 isolated from yeast are G418 resistant as expected, but cells bearing either of the 2 anomalous plasmids are sensitive to 200 μgml^{-1} G418. This suggests that the mutations or rearrangements are stable in that they were not reverted by the mutational effects sometimes associated with the transformation procedure.

1.3.1.5 - Re-transformed G418-sensitive yeast still give brighter signal outputs

FF18984 cells re-transformed with the altered reporter plasmids were tested for their ability to induce the reporter in response to 0.005% MMS, compared with cells carrying the normal pWDH445. After 15 hours incubation with MMS, fluorescence and scatter measurements were made and brightness values determined. **Figure 4** depicts the brightness signals from both cells bearing normal pWDH445 (darker bars) and cells conveying the altered plasmids (lighter bars). As previously, the lighter bars for the transformed cells are significantly larger than the corresponding darker bars. The modified plasmids gave rise to a 3.3 fold increase in the untreated brightness signal and a 4.6 fold increase in the MMS-induced signal, compared with cells bearing the unchanged pWDH445. Overall these increases had little effect on the induction ratio, producing only a slight decrease from 5.5 to 5.2 in

cells bearing modified plasmid. However, the increase in brightness signal with the re-transformants was not as great as for the original variants isolated.

1.3.2 Restriction analysis of pWDH445 and its brighter derivatives

It was hypothesised that there was a link between the enhancement of brightness signal and the loss of G418 resistance, which suggested a change in or the loss of the *kan*MX module. Investigation of this required the use of diagnostic restriction digestions.

A restriction map of pWDH445 defined in this study is shown in **Figure 5.**

1.3.2.1 - *Xba* I digestion revealed size differences in the rearranged plasmids

The *Bam*H I and *Asc* I double digestion employed previously to confirm the presence of the intact *HO-RAD54* promoter-*yEGFP* module also revealed that one rearranged plasmid was larger than pWDH445 whilst the other was smaller. *Xba* I digestion was used to confirm the size changes in the rearranged plasmids after fractionation of the DNA fragments by agarose gel electrophoresis. From the map, *Xba* I digestion to completion was expected to yield 2 bands in a doublet formation at ~6 kb, for pWDH445 and plasmid isolated from cells exhibiting normal brightness. In **Figure 6**, lanes 1 and 3 represent the fragments created by *Xba* I digestion of pWDH445 and plasmid from cells of normal brightness, separated on a 1% agarose gel and labelled with ethidium bromide. Both lanes show identical bands in a doublet formation at an approximate size of 6 kb (lane 5 contains 500 bp ladder molecular weight marker DNA, with the largest fragment at 5 kb). Lane 4 represents the *Xba* I fragments for the rearranged plasmid that appeared to be smaller in the *Bam*H I – *Asc* I double digestion. The smaller size is also reflected in the replacement of the larger band from the doublet (~6 kb) with a band at ~4.4 kb, giving a vector size of ~10.4 kb as opposed to the 12 kb of pWDH445. Lane 2 shows the *Xba* I fragments for the other rearranged plasmid that appeared to be larger than pWDH445 in the *Bam*H I – *Asc* I digestion. As with the smaller rearranged plasmid, the larger band from the 6kb doublet is lost, but replaced not only with a band of ~4.4 kb, but also 2 further new bands at ~3.2 and 3.0 kb. This would suggest a vector size of ~16.6 kb, an increase of ~4.6 kb compared with pWDH445. This suggests that the brightness increase in cells carrying the rearranged plasmids was not purely a vector size-related phenomenon

(commonly there is an inverse relationship between size and copy number). It is also suggested that both rearrangements occur in the same half of the vector since the larger of the ~6 kb bands is lost in each case. The larger band represents the half of the plasmid bearing *Amp*^{r}, *kan*MX3, and *yEGFP*. This further corroborated the suggestion from the *Bam*H I – *Asc* I digestion, that the *HO-RAD54* promoter-*yEGFP* module was intact after rearrangement.

1.3.2.2 - *Sca* I digestion further limited the region of rearrangement

pWDH445 contains four recognition sites for the *Sca* I restriction enzyme and hence *Sca* I digestion should result in the production of four fragments. The four fragments should be represented by bands at approximately 4.7, 4.1, 1.9, and 1.3 kb after electrophoretic separation. **Figure 7** shows a picture of the ethidium bromide stained 1% agarose gel used to fractionate the *Sca* I fragments from pWDH445 (lane 2), the larger rearranged plasmid (lane 3), re-isolated pWDH445 (lane 4), and the smaller rearranged plasmid (lane 5). Lanes 2 and 4 show the same patterns of bands, with a doublet between 4 and 5 kb (representing the 4.7 and 4.1 kb fragments) and single bands at ~1.8 kb and 1.3 kb. Neither of the rearranged plasmids (lanes 3 and 5) exhibit the 1.3 kb *Sca* I band, the production of which requires the *Sca* I recognition site within *kan*MX3 to be intact. The ~1.8 and 4.1 kb bands are present in all 4 of the plasmids digested, implying that no alteration had occurred in the region containing *HO*, *URA3*, the 2 micron origin, or *Amp*^{r}. Hence, the portion of the vector in which the rearrangements could have occurred was further limited to the region between the unique *Asc* I recognition site and the *Sca* I recognition site in *Amp*^{r}, including the entire *kan*MX3 module.

1.3.2.3 - The *Pst* I cleavage site within the *kan*MX3 module is lost in the rearranged plasmids

Once the region of the plasmid incorporating the rearrangements was established, it was necessary to test restriction enzymes with cleavage sites in this section. The *Pst* I cleavage site occurs 7 times in pWDH445, though only 6 bands were expected to be detectable on an ethidium bromide stained 1% agarose mini-gel after electrophoretic separation of the *Pst* I fragments. The seventh fragment was too small to be detected under these conditions. Approximate sizes for the 6 bands were 4.2, 2.5, 2.2 or 1.6, 1.4, 1.3, and 0.9 kb. The fragment expected to be detected at 2.2

or 1.6 kb should result from cleavage at the sites within *HO* and *URA3*, but the size is dependent upon the orientation of the *URA3* gene.

Figure 8 shows a picture of the ethidium bromide stained gel in which the fragments from *Pst* I digested plasmid were separated. pWDH445 and re-isolated pWDH445 were loaded into lanes 2 and 4, respectively, whilst the larger and smaller rearranged plasmids were loaded into lanes 3 and 5, respectively. All four plasmids exhibit a 1.6 kb band suggesting that the *URA3* gene is transcribed in the opposite direction to the *HO-RAD54* promoter-*yEGFP-kan*MX3 cassette. The 2 bands involving the *Pst* I cleavage site in the *kan*MX3 module are the 2.5 and 0.9 kb fragments. Only lanes 2 and 4, containing digested pWDH445 and digested re-isolated pWDH445, produced bands at 2.5 and 0.9 kb (the 0.9 kb band is not visible in **Figure 8**) whilst these were absent for both rearranged plasmids (lanes 3 and 5). Loss of the 2.5 kb and 0.9 kb bands from both of the rearranged plasmids suggests that a rearrangement had occurred within *kan*MX, causing loss of the *kan*MX *Pst* I cleavage site. *Pst* I digestion of both of the rearranged plasmids produced a novel 1.5 kb band not present in the pWDH445 digestions. If only the *Pst* I cleavage site in *kan*MX3 had been altered in such a way as to prevent its cleavage, the appearance of a ~3.5 kb band would be expected (combination of the 2.5 and 0.9 kb fragments). However, recombination between the direct repeat elements flanking the Kan^r gene in the *kan*MX3 module could result in the loss of a 2 kb portion of the 3.5 kb fragment. This may account for the 1.5 kb band detected in lanes 3 and 5.

1.3.2.4 - Two out of three *Pvu* I cleavage sites were lost in both rearranged plasmids

There are four cleavage sites for the *Pvu* I restriction endonuclease in pWDH445, 3 of which lie within the *kan*MX3 module. The four fragments expected were of the sizes ~9, 1.2, 1.1, and 0.7 kb. The four plasmids were digested with *Pvu* I and the resulting fragments were fractionated on a 1% agarose gel by electrophoresis. The digested plasmids were loaded onto the gel with stock pWDH445 in lane 1, the larger rearranged plasmid in lane 2, isolated pWDH445 in lane 3, and the smaller rearranged plasmid in lane 4. **Figure 9** shows a picture of the ethidium bromide stained *Pvu* I fragments. Both lanes 1 and 3 exhibit all four of the expected bands whilst lanes 2 and 4 reveal different band patterns. The smaller rearranged plasmid in

lane 4 showed the large band, representing the bulk of the plasmid, and a band at 1.1 kb that all four plasmids exhibit. The 1.1 kb band results from cleavage by *Pvu* I in the *Amp*^{r} and *Kan*^{r} genes, suggesting that the *Pvu* I cleavage site at the 5' end of *kan*MX3 was intact. However, neither rearranged plasmid shows bands representing the 1.2 and 0.7 kb *kan*MX3 internal fragments. This suggests that a loss of DNA within the *kan*MX3 module has occurred. The larger rearranged plasmid also showed 2 novel bands at 1 kb and ~3.5 kb.

1.3.2.5 - *Sac* I does not liberate ~2.1 kb of *kan*MX3 from the rearranged plasmids

There are 2 *Sac* I cleavage sites in pWDH445, one in each of the direct repeat sequences flanking *Kan*^{r} in *kan*MX3. Thus, 2 bands representing 2 fragments of ~2.1 kb and ~10 kb should be produced by *Sac* I digestion of pWDH445. The 2.1 kb fragment is released from *kan*MX3 whilst the larger fragment represents the remainder of the plasmid. The four plasmids were digested with *Sac* I restriction endonuclease and the resulting fragments were separated electrophoretically in a 1% agarose gel. **Figure 10** is a picture of the ethidium bromide stained gel with pWDH445 in lane 1, the larger rearranged plasmid in lane 2, isolated pWDH445 in lane 3, and the smaller rearranged plasmid in lane 4. Only lanes 1 and 3 exhibit the expected 2.1 kb fragment released from *kan*MX3, whilst lanes 2 and 4 both show only a single band representing uncut plasmid. In conjunction with the other evidence discussed above, this suggests that both rearranged plasmids have lost a portion of *kan*MX3 between and including the *Sac* I cleavage sites in the direct repeat sequences.

1.3.3 Enzymic removal of *kan*MX3 from pWDH445

The nature of the investigations described above is such that a considerable amount of time could have been consumed in attempting to precisely define the rearragement(s). Since a significant rearrangement was defined within the *kan*MX module, it was decided to re-create this modification by enzymically excising *kan*MX3. This would provide a means to test the hypothesis that the enhancement of brightness from cells bearing the rearranged plasmids was due to the loss of part of the *kan*MX3 module.

1.3.3.1 – *Sac* I digestion of pWDH445 followed by re-ligation

Stock pWDH445 was digested with *Sac* I to completion, in order to remove a ~2.1 kb fragment from the *kan*MX3 module. A 3 µl aliquot of *Sac* I-digested pWDH445 was loaded onto a 1% agarose gel and the fragments separated by electrophoresis in order to check the success of the digestion (data not shown). After heat inactivation of the *Sac* I enzyme by incubation at 65°C for 20 minutes, digested pWDH445 was then re-ligated. Ligations were performed at 3 different concentrations of digested plasmid DNA (undiluted, 1 in 10 fold dilution, and 1 in 100 fold dilution) by incubation with T4 DNA ligase at 16°C overnight (method **1.2.5.4.2**). Dilutions of digested DNA were used in order to promote intramolecular ligation events. A 10 µl aliquot of each ligation mix was used to transform *E. coli* and transformants were selected by overnight incubation at 37°C on LB plates containing ampicillin. Ampicillin resistant transformants were replica-plated onto LB plates containing kanamycin. After overnight incubation on kanamycin plates, very few colonies were able to grow.

10 ampicillin resistant colonies were selected, including one transformant that was also kanamycin resistant, and incubated with shaking in LB broth containing ampicillin at 37°C overnight. Plasmid preparations were made from these cultures using the method described in method **1.2.5.3.2** and the isolated DNA digested with *Xba* I along with normal pWDH445. Fragments were separated by electrophoresis in a 1% agarose gel and the ethidium bromide stained gel is shown in **Figure 11**. Lanes 1 and 13 show the bands representing the *Xba* I fragments from pWDH445, though overloading makes the 6 kb fragment difficult to distinguish. Lane 7 contained a 500 bp ladder molecular weight marker with the laregest band at 5 kb, demonstrating that the bands in lanes 1 and 13 were ~6 kb. Lane 10 contained DNA from the kanamycin resistant colony and reveals the same 6 kb band as the controls in lanes 1 and 13. The remainder of the lanes contained DNA from kanamycin sensitive colonies and all exhibit a band at ~6 kb, but also a second band at ~4.5 kb. This is the same banding pattern as that seen for *Xba* I-digestion of the smaller rearranged plasmid (see lane 4, **Figure 6**).

1.3.3.2 - The new plasmid is brighter than pWDH445 in yeast

FF18984 cells were transformed with DNA from preparations of the new plasmid using the method described in **1.2.5.2.1**. Transformants were selected for by incubation on SD plates lacking uracil and the auxotrophic markers were checked. 10 transformants were picked for assessment of brightness in response to 0.005% MMS. Incubation with MMS lasted 15 hours before measurement of fluorescence and scatter. The average brightness values determined for cells bearing the new plasmid (red bars) are compared with those from cells carrying pWDH445 (blue bars) in **Figure 12.** The brightness values for the cells carrying the plasmid with part of *kan*MX3 enzymatically excised (**Figure 12**) were comparable with those of cells bearing the spontaneously modified plasmid isolated from yeast. Untreated cells bearing the new plasmid were found to be 4 fold brighter than those conveying pWDH445, whilst the brightness increased 5 fold for cells treated with MMS. However, the induction ratio was reduced in the brighter cells due to the increase in the constitutive level of brightness.

1.3.3.3 Plasmids according to the present invention

Data is presented in Example 2

1.4 SUMMARY

1. Transformation of FF18984 with pWDH445 generated some transformants with a 12 fold brighter constitutive signal and a 9 fold brighter MMS-induced signal than typical pWDH445-bearing FF18984 cells. The induction ratio was halved in the brighter cells.
2. Bright transformants were unable to grow on medium containing G418, suggesting an alteration within the *kan*MX3 module of pWDH4454.
3. Double restriction of plasmids isolated from the brighter transformants with *Bam*H I and *Asc* I produced the same size bands as pWDH445, suggesting that the *HO-RAD54* promoter-*yEGFP* module was unchanged. However, one rearranged plasmid was larger than pWDH445 whilst another was smaller.
4. Yeast cells (FF18984) re-transformed with rearranged plasmid were sensitive to G418, confirming that the change in brightness was due to the plasmid change , as opposed to a chromosomal mutation.

5. Untreated and MMS-induced re-transformed yeast cells were 3.3 fold and 4.6 fold brighter than pWDH445-carrying cells, respectively. However, there was little change in induction ratio between cells conveying pWDH445 or rearranged plasmid.
6. Digestion with *Xba* I corroborated the finding from *Bam*H I-*Asc* I double digestion that one plasmid was larger and one smaller than pWDH445. The fragment altered by the rearrangements carries *Amp*^{r}, *kan*MX3, and *yEGFP*.
7. *Sca* I digestion suggested that no rearrangement had occurred in the region bearing *HO*, *URA3*, the 2 micron origin, and part of *Amp*^{r}. In conjunction with the findings from the *Bam*H I-*Asc* I and *Xba* I digestions, rearrangement must have occurred between the unique *Asc* I cleavage site and the *Sca* I cleavage site in *Amp*^{r}, in a region incorporating the entire *kan*MX3 module.
8. The 2 bands formed by *Pst* I digestion if the *Pst* I site in *kan*MX3 is intact are not present for either of the rearranged plasmids.
9. 2 of 3 *Pvu* I cleavage sites in *kan*MX3 are not present in the rearranged plasmids suggesting loss of DNA within the *kan*MX3 module.
10. *Sac* I does not release the expected ~2.1 kb *kan*MX3 fragment from the rearranged plasmids.
11. A ~2.1 kb fragment of *kan*MX3 was cleaved from pWDH445 using *Sac* I before religation of the *Sac* I sticky ends. Yeast cells transformed with the new plasmid were as bright as the yeast cells bearing spontaneously rearranged plasmids.

1.5 DISCUSSION

Rearrangements of pWDH445 (to form recombinant vectors according to the first aspect of the invention) occurred, probably in a transformation-dependent manner given that transformation is known to be a mutagenic process, generating brighter than normal transformants. These bright transformants were found to be sensitive to growth on medium containing 200 $\mu g ml^{-1}$ geneticin, despite pWDH445 bearing a *kan*MX module incorporating the gene encoding the aminoglycoside phosphotransferase that confers resistance to geneticin on yeast cells. This suggested that mutation or loss of the *kan*MX module had occurred, leaving the cells sensitive to geneticin. It was anticipated that *kan*MX had probably been lost from the plasmid,

since a deletion event would reduce the size of the plasmid, increasing its stability and hence copy number.

However, after isolation of plasmid DNA from the bright transformants and digestion to release the *HO-RAD54*promoter-*yEGFP* cassette, it was clear from the bands representing the DNA fragments separated by gel electrophoresis that whilst one bright transformant carried a smaller reporter plasmid, another conveyed a larger derivative of pWDH445. This ruled out the hypothesis that the enhanced reporter output was a plasmid size-related phenomenon and thus, demanded more detailed investigation. The outcome of further restriction analysis of the rearranged plasmids revealed that *kan*MX was lost in both the smaller and larger plasmids, and the larger plasmid gained several kb of DNA.

The smaller plasmid was probably generated by recombination between the direct repeats that flank the kan^r gene and promoter sequences within the *kan*MX module. The *kan*MX fragment released by *Sac* I digestion is ~2.1 kb which is approximately the size of fragment lost by the small plasmid.

It has been previously shown that bacterial sequences borne by shuttle vectors can be toxic to heterologous host organisms and that such sequences unnecessary for maintenance in *S. cerevisiae* can be automatically eliminated by recombination events. Since all pWDH445 transformants were primarily selected for their ability to grow on SD medium lacking uracil, the *kan*MX module was unnecessary for plasmid maintenance.

Seemingly the enhancement of reporter brightness is not related to a reduction in size and hence increase in copy number, since one of the rearranged plasmids was shown to have increased in size. This suggests that the constitutive expression of the aminoglycoside phosphotransferase driven by the promoter from the translation elongation factor 1α (*AgTEF*) from the filamentous fungus *Ashbya gossipyii*, affects the expression of yEGFP from the *RAD54* promoter in some way. The *kan*MX module is immediately downstream of the *HO-RAD54*promoter-*yEGFP* cassette with only a direct repeat sequence (465 bp) separating *yEGFP* from *AgTEF*. While we do

not wish to be bound by any hypothesis, it is conceivable that the omnipresent (due to the constitutive expression) transcription complex for the aminoglycoside phosphotransferase interferes with termination of *yEGFP* transcription, resulting in imperfect transcripts and hence nascent yEGFP polypeptide that is incapable of legitimate folding to produce mature GFP. Loss of *kan*MX would remove the interference and permit a greater proportion of yEGFP to fold correctly and hence fluoresce, in both constitutive and induced reporter states.

Interference of efficient transcription of *yEGFP* might result from the conformation of the plasmid. The size and sequence of pWDH445 bearing *kan*MX might be such that the plasmid assumes a conformation restricting access of components of the transcriptional complex to the *RAD54* promoter. However, loss of *kan*MX regardless of the mechanism could result in alteration of the conformation, due to the change in size and sequence, leading to greater accessibility of the *RAD54* promoter. In this case, it must be that pWDH445 is of the critical size allowing it to assume the restrictive conformation, and hence any change in size, either increase or reduction, alters the conformation sufficiently.

An alternative explanation for the enhanced brightness in the absence of *kan*MX is the greater availability of general transcription factors. Since the aminoglycoside phosphotransferase encoded within *kan*MX is constitutively expressed, it must have a constant requirement for general transcription factors. Thus, the availability of such factors for transcription from the *RAD54* promoter is reduced in the presence of *kan*MX, and removal of this module eliminates the constraints on the reporter cassette. This is most likely only a "local" deficiency in the volume occupied by the plasmid, and might reflect a higher affinity for the transcription factors in the *AgTEF* promoter.

Finally, *kan*MX related interference with reporter expression might occur post-transcriptionally. Both yEGFP and the aminoglycoside phosphotransferase are heterologous proteins since GFP originates from *Aequorea victoria* and Kan^r from the *E. coli* transposon *Tn*903. It is possible that the 2 foreign proteins interact, preventing efficient protein folding and maturation of yEGFP. Loss of *kan*MX would result in loss of the illegitimate interaction, increasing the proportion of fluorescent yEGFP.

The mechanism by which *kan*MX is lost and reporter output is enhanced is essentially irrelevant to the commercial development of the reporter (as long as the response remains predictable), since a brighter reporter is obviously beneficial. The *kan*MX module was employed as a convenient selectable marker for research purposes, but stringent rules governing the release of genes conferring antibiotic resistance would require its displacement for commercialisation.

If the *kan*MX effect seen with pWDH445 is purely a plasmid-related phenomenon then the reporter cassette could be re-cloned into a different plasmid backbone, in order to determine if the effect is specific to pWDH445. Replacement of *kan*MX with a different gene conferring antibiotic resistance, such as hygromycin B (hph; hphMX), nourseothricin (nat; natMX), and bialaphos (pat; patMX), would resolve whether the effect is specific to *kan*MX on pWDH445. An intergenic region could be inserted between *yEGFP* and *kan*MX on pWDH445 to determine whether the effect is caused by the proximity of the *AgTEF* promoter of *kan*MX to the termination codon of *yEGFP*. Assessment of these new vectors would be by constitutive and induced brightness values when borne by the FF18984 strain. Bright transformants have been isolated from other yeast strains though plasmid DNA has not been isolated and assessed in the same way as from bright FF18984 cells. However, it is anticipated that similar rearrangements would be detected in plasmid isolated from alternative strains, which would discount a strain-specific effect.

EXAMPLE 2

2.1 Introduction

A yeast DNA repair reporter assay termed the GreenScreen® assay (GSA) is described. This assay represents a preferred method of detecting for the presence of an agent that causes DNA damage according to the third aspect of the invention.

We describe the simple, robust assay protocol, the development and a validation study; results of which indicate that compounds giving positive responses have a high likelihood (96%) of corresponding positive responses in one or more of the regulatory tests (Ames, Micronucleus and Mouse Lymphoma Tests or carcinogenicity bioassays). The false positive rate is extremely low. The endpoint of the test reflects the typically eukaryotic chromosomes and DNA metabolising enzymes of *Saccharomyces cerevisiae*. The capacity for metabolic activation (MA) is limited compared to the mammalian liver or its extracts, but the assay does detect a subset of compounds that would require MA in existing genotoxicity tests. The GSA detects a different spectrum of compounds to bacterial genotoxicity assays and thus, together with an *in silico* Structure Activity Relationship (SAR) screen, and possibly a high throughput bacterial screen, would provide an effective preview of the regulatory battery of genotoxicity tests.

Here we describe a protocol suited to the use of robotic liquid handling systems and present simple data handling protocols that provide clear graphical output.

2.2 Materials and Methods

2.2.1 Strains & Plasmids.

The *Saccharomyces cerevisiae* strain FF18984 (*MATa, leu2-3,112 ura3-52 lys2-1 his7-1*) was obtained from Francis Fabre (French Atomic Energy Commission (CEA), Fontenay-aux-Roses, France).

A FF18984 reporter strain containing a replicative plasmid (pGenRNR2) containing the upstream non-coding DNA sequence of the *RNR2* gene fused to the yeast-enhanced *Aequorea victoria* GFP gene. A control FF18984 strain containing

an identical plasmid except that 2 base pairs have been removed at the start of the GFP gene, such that no GFP is made.

2.2.2 Microplate preparation.

Assays were carried out in 96 well, black, clear-bottomed microplates (Matrix ScreenMates, Cat. No. 4929, Apogent Discoveries, USA). A number of alternative microplates were assessed, though the variable background absorbance and fluorescence both within and between plates from individual manufacturers were generally unacceptable, leading to the conclusion that only Matrix or Corning (BV, Netherlands: Cat. No. 3651) plates were appropriate for this assay. The assays were performed using a liquid handling robot (MicroLabS single probe, Hamilton GB Ltd., Birmingham. UK) in a protocol designed to set up 4 compounds per test on a single 96 well microplate in 30 minutes. Results for a subset of compounds have been reproduced using a Genesis 8-probe robot (Tecan UK Ltd. Theale. UK), which can set up a similar microplate in less than 5 minutes. Microplates can also be filled rapidly and effectively using a multi-channel pipette.

A microplate version of the assay has been previously reported (Afanassiev *et al.*, 2000), but different microplate layouts and controls have been used for this study, so more detail is presented here. The following standard protocol was followed. A 1 mM stock of the test chemical was prepared in 2% v/v aqueous DMSO, and used to make 2 identical dilution series across the microplate and a 'control' (see below). To achieve this, 150 microlitres of the test chemical solution were put into 2 microplate wells. Each sample was serially diluted by transferring 75 microlitres into 75 microlitres of 2% DMSO, mixing, and then taking 75 microlitres out and into the next well. This produced 9 serial dilutions of 75 microlitres each.

Controls were added as follows:

a. Compound alone, to provide information on compound absorbance/fluorescence
b. Yeast cultures diluted with 2% DMSO alone, to give a measure of maximum proliferative potential
c. MMS as a genotoxicity control: 'high' = 0.00125% v/v, 'low' = 0.0001875% v/v
d. Methanol as a cytotoxicity control: 'high' = 3.5% v/v, 'low' = 1.5% v/v.
e. Growth medium alone, to confirm sterility/lack of contamination

Stationary phase cultures of GenT01 and GenC01 were diluted to an optical density (OD_{600nm}) = 0.2 in double strength F1 medium (Billinton *et al.*, 1998). 75 microlitres of the yeast suspension were added to each well of the diluted chemical: GenT01 to one series and GenC01 to the second, and to appropriate standards and controls. After the plates were filled, they were sealed using either a gas permeable membrane (Breath-easy, Diversified Biotech, USA) or a plastic lid, and then incubated without shaking, overnight at 25°C.

2.2.3 Compounds chosen for the study.

MMS was used as a test compound to test the usefulness of recombinant vectors according to the present invention.

2.2.4 Data collection and handling.

Following overnight incubation, fluorescence and absorbance data were collected from the microplates. Two different microplate readers which combine fluorescence and absorbance functionality have been used, and comparable data were obtained. These were a Tecan Ultra-384 (Tecan UK Ltd.): excitation 485 nm / emission 535 nm with an additional dichroic mirror (reflectance 320 nm – 500 nm, transmission 520 nm – 800 nm), and a BMG PolarStar (BMG Labtechnologies, Germany): excitation 485 nm / emission 520 nm. Absorbance was measured through a 620 nm filter in both instruments. The data were transported into a Microsoft Excel spreadsheet, and converted to graphical data (see typical data in Figure 18). Data processing is minimal: absorbance data give an indication of reduction in proliferative potential and these data were normalised to the untreated control (=100% growth). Fluorescence data were divided by absorbance data to give 'brightness units', the measure of average GFP induction per cell. These data were normalised to the untreated control (=1). In this way, one can distinguish between a small number of highly fluorescent cells and a large number of weakly fluorescent cells. In order to correct for induced cellular autofluorescence and intrinsic compound fluorescence, the brightness values for the GenC01 strain were subtracted from those of GenT01. This makes visual assessment of the data more reliable. All the data were checked with

and without this correction, and the decision (see below), on whether or not a compound was classified as being genotoxic, was not affected.

2.2.5 Decision thresholds.

It is useful to have clear definitions of positive and negative results from routine assays and such definitions have been derived, taking into account the maximum noise in the system and data from chemicals where there is a clear consensus on genotoxicity and mechanism of action. Naturally it is also possible for users to inspect the numerical and graphical data and draw their own conclusions. For example an upward trend in genotoxicity data that did not cross the threshold might still distinguish two compounds. The decision thresholds were set as follows:

The cytotoxicity threshold is set at 80 % of the cell density reached by the untreated control cells. This is greater than 3 times the standard deviation of the background. A positive cytotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a final cell density lower than the 80% threshold. A strong positive cytotoxicity result positive (++) is concluded when either (i) three or more compound dilutions produce a final cell density lower than the 80% threshold or (ii) at least one compound dilution produces a final cell density lower than a 50% threshold. A negative result (-) is concluded when no compound dilutions produce a final cell density lower than the 80% threshold. The lowest effective concentration (LEC) is the lowest test compound concentration that produces a final cell density below the 80% threshold.

The compound absorbance control allows a warning to be generated if a test compound is significantly absorbing. If the ratio of the absorbance of the compound control well to a well filled with diluent alone is > 2, there is a risk of interference with interpretation. The cytotoxicity controls indicate that the yeast is behaving normally. The 'high' methanol standard should reduce the final cell density to below the 80% threshold, and should be a lower value than the 'low' standard.

The genotoxic threshold is set at a relative GFP induction of 1.3 (i.e. a 30% increase). This is greater than 3 times the standard deviation of the background. A positive genotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a

relative GFP induction greater than the 1.3 threshold. A strong positive genotoxicity result (++) is concluded if either (i) three or more compound dilutions produce a relative GFP induction greater than the 1.3 threshold or (ii) at least one compound dilution produces a relative GFP induction greater than a 1.6 threshold. A negative genotoxicity result (-) is concluded where no compound dilutions produce a relative GFP induction greater than the 1.3 threshold. The LEC is the lowest test compound concentration that produces a relative GFP induction greater than the 1.3 threshold. The genotoxic controls demonstrate that the strains are responding normally to DNA damage. The 'high' MMS standard must produce a fluorescence induction > 2, and be a greater value than the 'low' MMS standard. Anomalous brightness data is generated when the toxicity leads to a final cell density less than 30% that of the blank. Genotoxicity data is not calculated above this toxicity threshold. Compounds that tested negative for genotoxicity, were re-tested up to 10mM, or to the limit of solubility or cytotoxicity.

The compound fluorescence control allows a warning to be generated when a compound is highly auto-fluorescent. If the ratio of the fluorescence of the compound control well to a diluent filled well is >5, there is a risk of interference with interpretation. In these cases (four in this study), fluorescence polarisation can be used to distinguish GFP from other sources of fluorescence (Knight *et al.*, 2000, 2002). Both the Tecan and BMG instruments have this facility. Occasionally, compounds though not fluorescent themselves, induce cellular auto-fluorescence. This is apparent from rising brightness in the control (GenC01) strain in the absence of fluorescence from the chemical-only control. The routine subtraction of GenC01 from GenT01 data removes this interference from the data.

2.3 Results

2.3.1 pGen RNR2

Figure 19 illustrates results obtained in a yeast test strain transformed with pGen RNR2.

Brightness/fluorescence was induced by the genotoxic compound MMS. The inventors believe this compound activates the RNR2 regulatory element which in turn results in the expression of GFP and thereby produces a signal measurable according to the method of the invention

2.3.2. pGen RNR3

Figure 20 illustrates data as discussed in 2.3.1. save the yeast strain was transformed with pGen RNR3

EXAMPLE 3

This example provides a preferred protocol for carrying out the method of the invention using a plate assay procedure.

1) **PREPARING THE STARTER CULTURES.**

2 different strains are currently used per test: GenC01 (control strain) and GenT01 (the test strain). The cultures are inoculated and incubated in an orbital shaker at 200 rpm and 30 °C for three days (72 hours), after which the cells will be in 'stationary phase'. Culture flasks are then stored in a refrigerator (+ 4 °C) and can be used for tests over a period of up to seven days.

Each starter culture is prepared as follows :

Into a sterile 250 ml conical flask place -

10 ml sterile water
10 ml 2X F1 media (See Appendix 1)
1 x 250 µl colour coded aliquot of frozen cells

Seal flask with foam bung (taped to the flask) before incubating.

2) **PREPARING THE TEST COMPOUNDS.**

Standard solutions of the test compounds should be freshly prepared shortly before each test run. Unless a specific test concentration is required, the recommended top concentration of a test compound is 1 mM in 4 % DMSO v/v in water. It is necessary to ensure that the final concentration of test compound is made up in the same diluent as that supplied to the robot for serial dilutions. The diluent is 4 % DMSO v/v in water by default but can be substituted for water alone.

1 ml of test compound solution is required per 'test run' and there is sufficient capacity on a 96-well plate for 4 test runs. Therefore for each plate prepare 2 tubes each containing 1 ml of the respective test compound, label them, cover with black caps and leave in a fume cupboard until required. For reference record the details of the test compound, its concentration, dilution calculation and the diluent used.

3) **PREPARING THE CONTROL COMPOUNDS.**

The control compounds are prepared in 4% DMSO v/v in water to the following concentrations:

MMS High	=	0.002500 % v/v*
MMS Low	=	0.000375 % v/v*
Methanol High	=	7 % v/v*
Methanol Low	=	3 % v/v*

200 µl of each control chemical is required per plate (this includes a volume required to allow safe automated pipetting distance). Hence for a set of 4 plates, make up at least 800 µl of each control.

** By default all concentrations are halved when plated out, as a sample volume of 75 µl is combined with 75 µl of yeast culture producing a 50:50 dilution.*

MMS and Methanol prepared from 100% stock solution with a diluent of 4 % DMSO / water (v/v).

MMS:
5 µl 100 % MMS + 995 µl diluent = 0.5 % MMS Solution
Standard 1 (High): 20 µl 0.5 % MMS + 3980 µl diluent = 0.002500 % MMS
Standard 2 (Low): 450 µl 0.0025 % MMS + 2550 µl diluent = 0.000375 % MMS

Methanol:
Standard 1 (High): 280 µl + 3720 µl diluent = 7 % Methanol
Standard 2 (Low): 120 µl + 3880 µl diluent = 3 % Methanol

4) PREPARING THE CELLS.

Measure the optical density (at 600 nm) of the 'stationary phase' GenT01 and GenC01 starter cultures using a 1 ml disposable cuvette with a 1 cm path length and water as a comparison blank. Due to the high density of the starter cultures, dilute 20 fold (950 µl sterile water + 50 µl cell culture) to enable measurement in the linear range. Calculate the OD of the undiluted cultures (Y) using the equation below:

$$\text{OD of the starter culture } (Y) = \text{Measured OD} \times 20$$

Note: Once in cold storage (+ 4 °C) the cell density does not change significantly over the course of a week, and do not need to be re-measured before each day's use.

Prepare a fresh suspension of both GenT01 and GenC01 cells in 2X F1 media, at an initial OD of 0.2 units/ml. 4 ml of GenC01 and GenT01 cell suspensions are required per plate (3.3 ml plus dead volume). Therefore for 4 microplates the total volume of fresh cell suspension required is 16 ml for GenC01 and 16 ml for GenT01.

Use the following equation to calculate, V, the volume of starter culture required to prepare the new cell suspension:

$$V = \frac{0.2 \times \text{volume of fresh culture required (ml)}}{Y}$$

Y = Optical density (O.D) of starter culture

Transfer *V* ml of stock cell culture into a sterile 5ml glass test tube containing 4mls of 2x F1 media.

Therefore for 4 microplates 16 ml of GenC01 is split into 4 x 4 ml aliquots in (**blue** capped) test tubes, and 16 ml of GenT01 is split into 4 x 4 ml aliquots (**red** capped) test tubes. For a lower number of plates scale down accordingly.

6) PREPARING ADDITIONAL TUBES AND SOLUTIONS.

Place 3.5 ml of 90 % ethanol in a single tube.

Place 3.5 ml of "media only" (1xF1) in a single tube.

Place approx. 900 ml of 10 % 'Chloros' in a 1 L container as a disinfectant, and toxic chemical neutralising, solution into which caps and spent test tubes can be sterilised before washing, autoclaving and recycling.

7) SET-UP FOR THE PIPETTING ROBOT AND DECK LAYOUT.

Switching on and Software Set-up

i. SWITCH ON the equipment in the following order.

- The fume hood.
- Computer.
- MVP (Diluent Selector Unit) at mains plug.
- The robot using the switch on right hand side.
- load up 'Microlab Sampler Software'.

If the robot has been left filled with anything other than sterile water, purge the system with sterile water using a wash program before use. The following methods are available. To run use *File / Open* the click "Run Method", "Start" and follow the on-screen instructions.

Wash Needle/System.Ame	Washes 10 ml of a solution connected to MVP position 1 into a waste container placed in the 'Bleach wash' position.
Wash Station/Tubing.Ame	Washes the needle and "wash station" and all associated pump tubing for cycles of 10 s, from the "clean wash" reservoir to waste.

ii. The control program reads in the settings for the chosen diluent, sample volume, yeast volume and the volume carried over in each serial dilution for each plate. The default values are as follows.

PLATE	DILUENT	SMPVOL	YEASTVOL	DILVOL1	DILVOL2	DILVOL3	DILVOL4
1,	1,	75,	75,	75,	75,	75,	75
2,	1,	75,	75,	75,	75,	75,	75
3,	1,	75,	75,	75,	75,	75,	75
4,	1,	75,	75,	75,	75,	75,	75

To alter the values or check the current configuration open the file C:/Program Files/Hamilton/Microlab Sampler/Methods/4COMPOUND.lay in Notepad. Make the alteration then select *File / Exit* and only when prompted with "The text in the file has changed. Do you want to save the changes?" select *Yes* to resave the file.

iii. In the Microlab Sampler software select *File / Open* and open 4COMPOUND.lay

iv. Select "Run Method".

Loading the Robot Deck and Plating Out

i. Before commencing any method carefully clean the outside of the needle by hand with ethanol using a soft cloth.

ii. Load the required number of Matrix micro-plates onto the robot deck in the positions as shown on the screen.

iii. Load the GREEN RACK with the appropriate tubes containing yeast cultures, samples, standards, 90 % ethanol, plate blank and an addition one for waste volumes of the test compounds according to the loading sequence (see figure 21).

iv. Place the appropriate diluent solutions in position to the right hand side of the deck with tube lines connected to the corresponding MVP selector positions. (Position 1 by default).

v. Place the 'clean wash' tube in a container of sterilised water (approx. 300 ml per plate).

vi. Ensure the 'dirty water' receptacle has sufficient capacity to collect the wash solution supplied.

vii. Remove all tube caps and place in a suitable disinfectant / compound neutralising solution (i.e. 10 % Chloros).

viii. Select *START* in the 'Run Method' software.

ix. The method will now run taking approximately 30 minutes to set up each plate according to the layout described in figure 22.

x. Once the method is completed empty the remaining contents of the test tubes in the Green Rack into a sealable plastic bag containing a suitable absorbent material. Seal, autoclave and dispose of the bag according to local regulations. Place the empty glass test tubes into the Chloros solution for sterilisation and decontamination before subsequently washing and recycling.

8) INCUBATING THE PLATES.

Once the plate is complete cover with breathable membrane or plate lid and label clearly. Shake plate vigorously for 30 seconds on microtitre plate shaker (to fully mix the contents of each well) and then incubate at 25 °C, unshaken, for approximately 16 hours (or overnight).

9) ROBOT MAINTENANCE

Wash through the pipetting robot with sterile water. (See section 7 i for details). When leaving for long periods (i.e. > 2 days) leave the system filled with 70 % ethanol v/v in water. Approximately once a week wash through the system with a suitable disinfectant (i.e. dilute Chloros solution) and then thoroughly with sterile water so that future user's solutions are not contaminated.

Clean the outside of the needle with a soft cloth soaked in ethanol.

Position the needle such that the needle tip is left immersed in sterile water contained in a test tube in the Green Rack, in position A1. (Once switched off the needle and robot arm can be moved manually taking care not to bend or damage the needle. Its position will re-set automatically when commencing a new method.)

10) PLATE READING AND DATA ANALYSIS

For plate reading and analysis refer to the protocol appropriate for the plate reader used. i.e. Tecan Ultra or BMG Polar Star.

11) DISPOSAL

When all results processing is completed, contain the used micro-plates within a sealed plastic bag containing a suitable absorbent material. Seal, autoclave and dispose of according to local regulations.

CLAIMS

1. A recombinant vector comprising a recombinant DNA molecule comprising a comprising a RNR regulatory element operatively linked to a DNA sequence that encodes a light emitting reporter protein and a DNA vector characterised in that the vector comprises an origin of replication; at least one selectable maker; and when used to transform a cell, does not alter the sensitivity of the cell to geneticin.

2. A recombinant vector according to claim 1 comprising the vector of Figure 5 with a deletion, substitution or addition that disrupts the function of the KanMx3 gene; and an RNR regulatory element in place of the RAD54 regulatory element.

3. A recombinant vector according to claim 1 comprising the vector of Figures 15 or 16 or functional derivatives thereof.

4. A recombinant vector according to claim 1 wherein the recombinant DNA molecule comprising a yeast RNR regulatory element operatively linked to a DNA sequence that encodes a light emitting reporter protein

5. The recombinant vector according to any preceding claim wherein the light emitting reporter protein is Green Fluorescent Protein and light-emitting derivatives thereof.

6. The recombinant vector according to claim 5 wherein the recombinant DNA molecule has a DNA sequence encoding the S65T derivative of Green Fluorescent Protein.

7 The recombinant vector according to claim 5 wherein the recombinant DNA molecule has a DNA sequence encoding a Yeast Enhanced derivative of Green Fluorescent Protein.

8. The recombinant vector according to any preceding claim, wherein the vector is designed to autonomously replicate in a cell.

9. The recombinant vector according to any preceding claim, wherein the vector contains DNA from the 2μ plasmid.

10. The recombinant vector according to any preceding claim, wherein the vector contains DNA encoding for part of the HO gene from chromosome IV of a yeast.

11. The recombinant vector according to claim 1, wherein the DNA vector is a PFA vector.

12. A cell containing a recombinant vector according to any one of claims 1 to 11.

13. A cell containing a recombinant vector according to claim 2 or 3.

14. The cell according to claims 12 or 13 wherein the cell is a yeast.

15. The yeast according to claim 14, wherein the yeast is *Saccharomyces cerevisiae.*

16. The yeast according to claim 15 which is FF18984 or Y486 in haploid form.

17. A method of detecting the presence of an agent that causes or potentiates DNA damage, the method comprising subjecting a cell according to claim 12 to an agent and monitoring the expression of the light emitting reporter protein from the cell, wherein an increase of the expression in the presence of the agent indicates that the agent causes or potentiates DNA damage.

18. The method according to claim 17, wherein the agent is further screened to assess whether it is safe to expose a living organism to the agent.

19. The method according to claim 17, wherein the agent is a candidate medicament, food additive or cosmetic.

20. The method according to claim 17, wherein the agent is a contaminant of water supplies.

21. The method according to claim 17, wherein the agent is a contaminant of industrial effluents.

22. The method according to claim 17, wherein expression of the light emitting reporter protein is measured from a whole cell.

23. The method according to claim 22, wherein the light emitting reporter protein is Green Fluorescent Protein.

24. The method according to claim 23 comprising growing cells transformed with a recombinant vector according to claim 5, incubating the cells with the agent for a predetermined time and monitoring the expression of the Green Fluorescent Protein directly from a sample of the cells.

25. The method according to any one of claims 17 - 24 wherein the cells are grown in a low fluorescence growth medium.

26. The method according to claim 25 wherein the low fluorescence growth medium is F1 medium.

FIG. 1

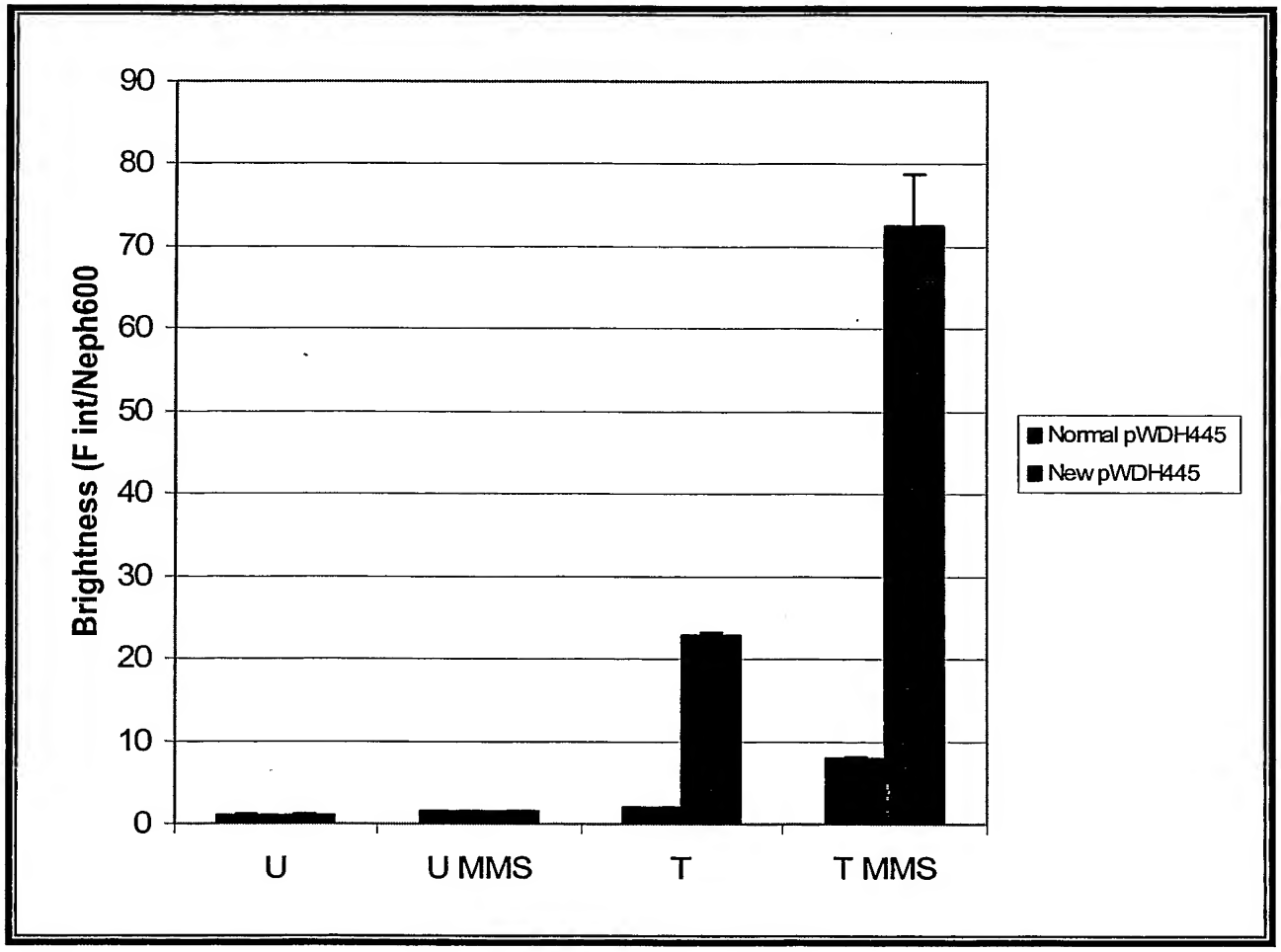

Brightness (F int/Neph600
90
80
70
60
50
40
30
20
10
0
U
U MMS
T
T MMS
Normal pWDH445
New pWDH445

FIG.2

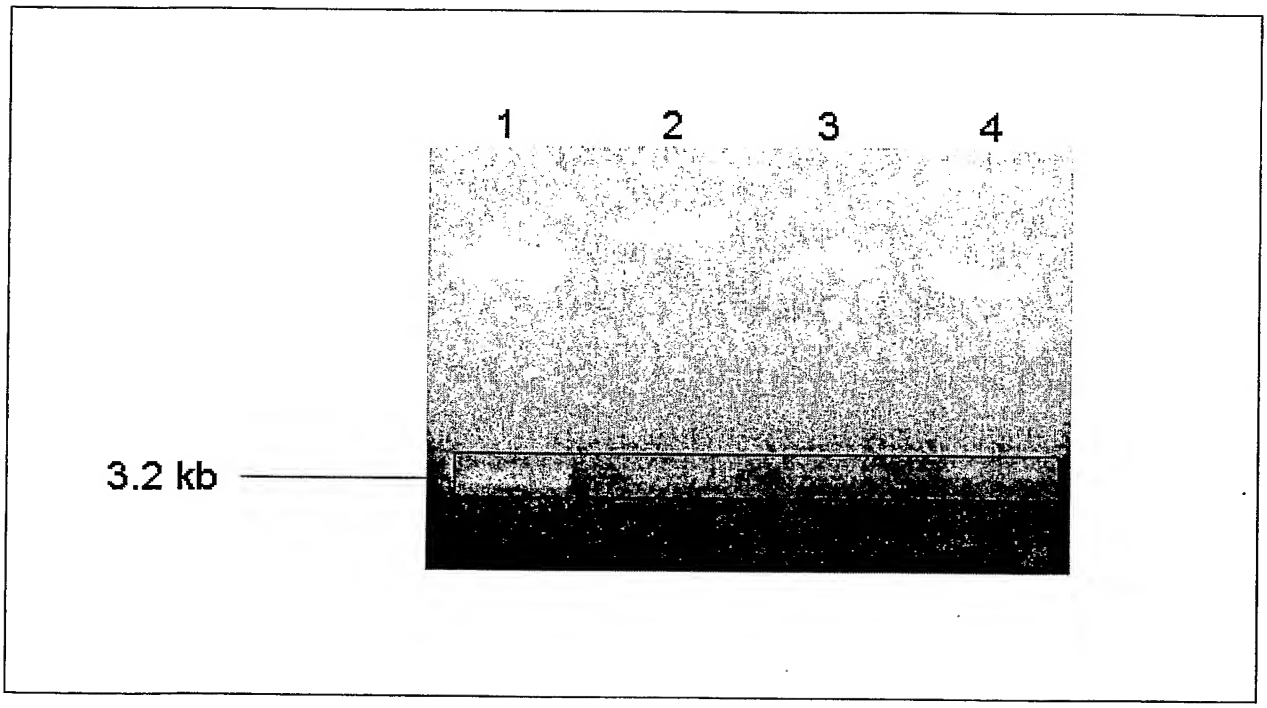


FIG. 3

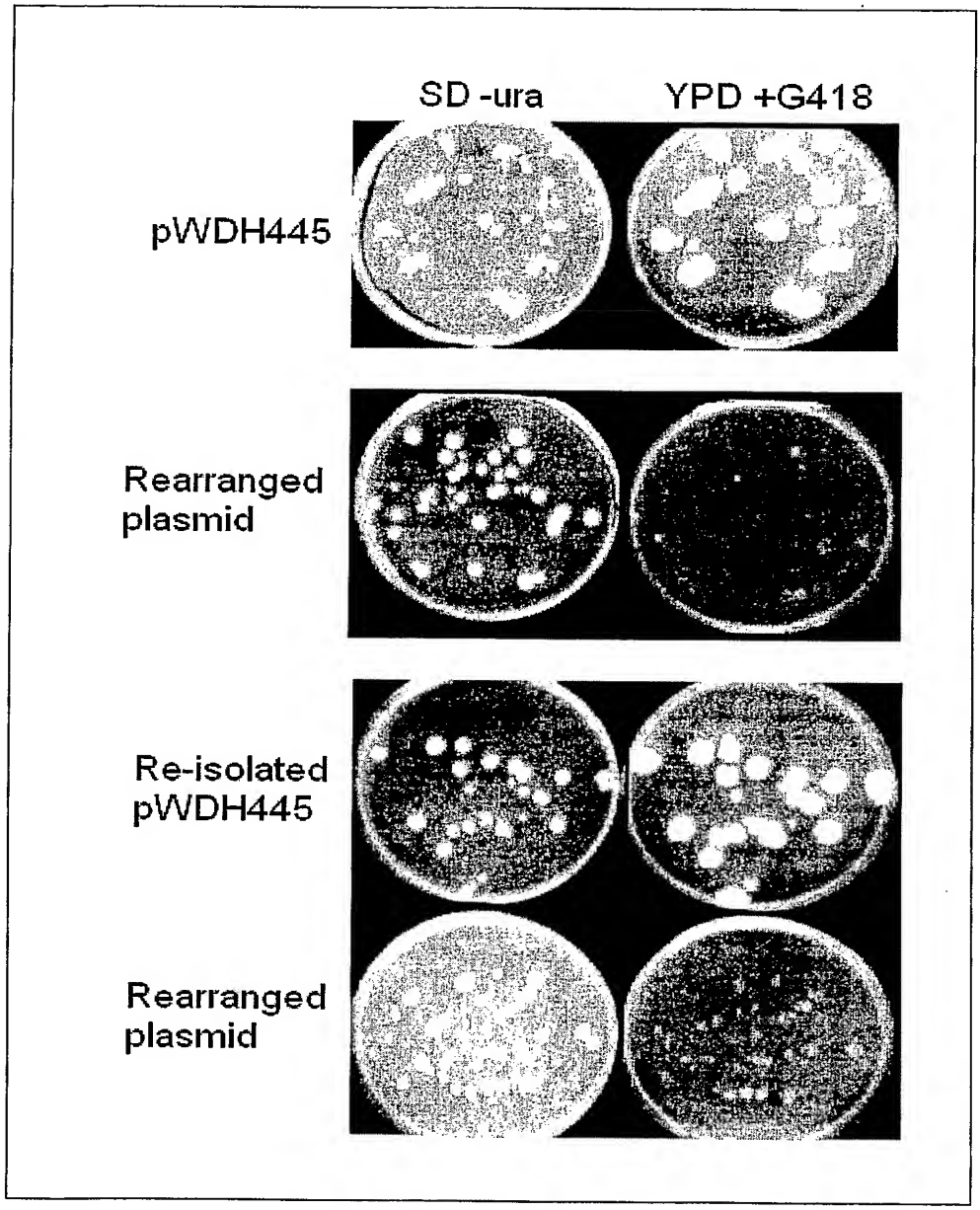

SD -ura
YPD +G418
pWDH445
Rearranged plasmid
Re-isolated pWDH445
Rearranged plasmid

FIG. 4

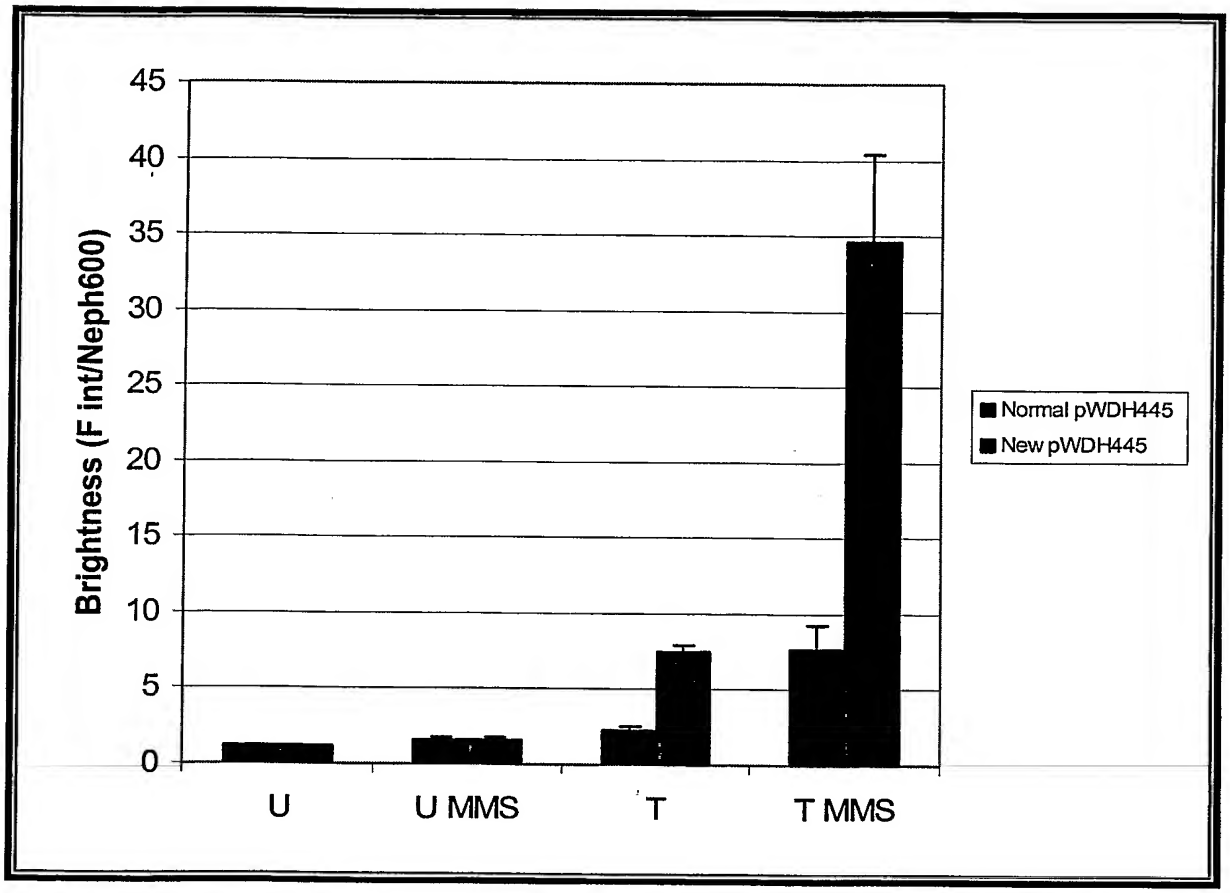

Brightness (F int/Neph600)
45
40
35
30
25
20
15
10
5
0
U
U MMS
T
T MMS
Normal pWDH445
New pWDH445

FIG. 5

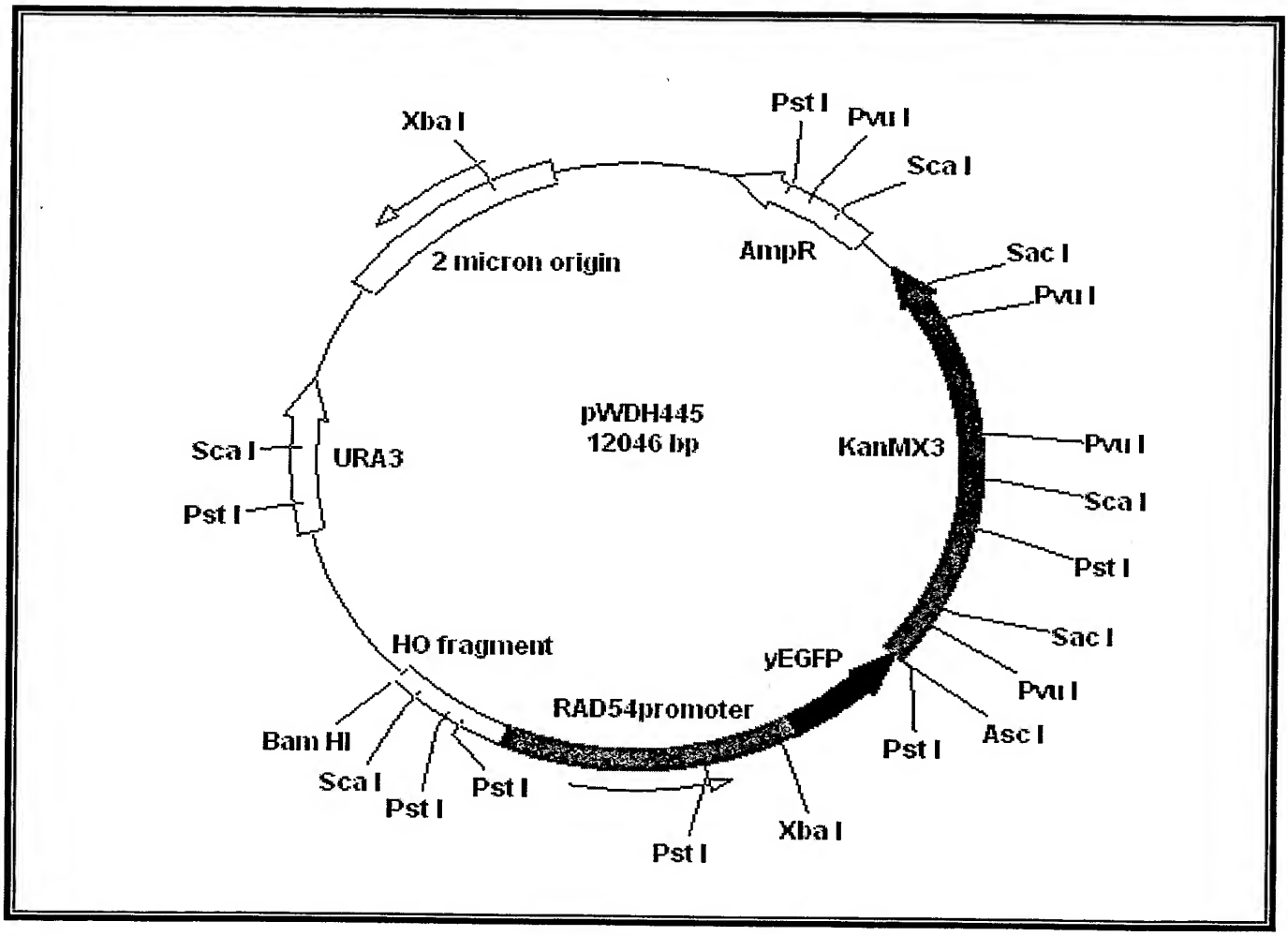

Xba I
Pst I
Pvu I
Sca I
2 micron origin
AmpR
Sac I
Pvu I
pWDH445
12046 bp
Sca I
URA3
KanMX3
Pvu I
Sca I
Pst I
Pst I
Sac I
HO fragment
yEGFP
Pvu I
RAD54promoter
Asc I
Pst I
Bam HI
Sca I
Pst I
Pst I
Xba I
Pst I

FIG. 6

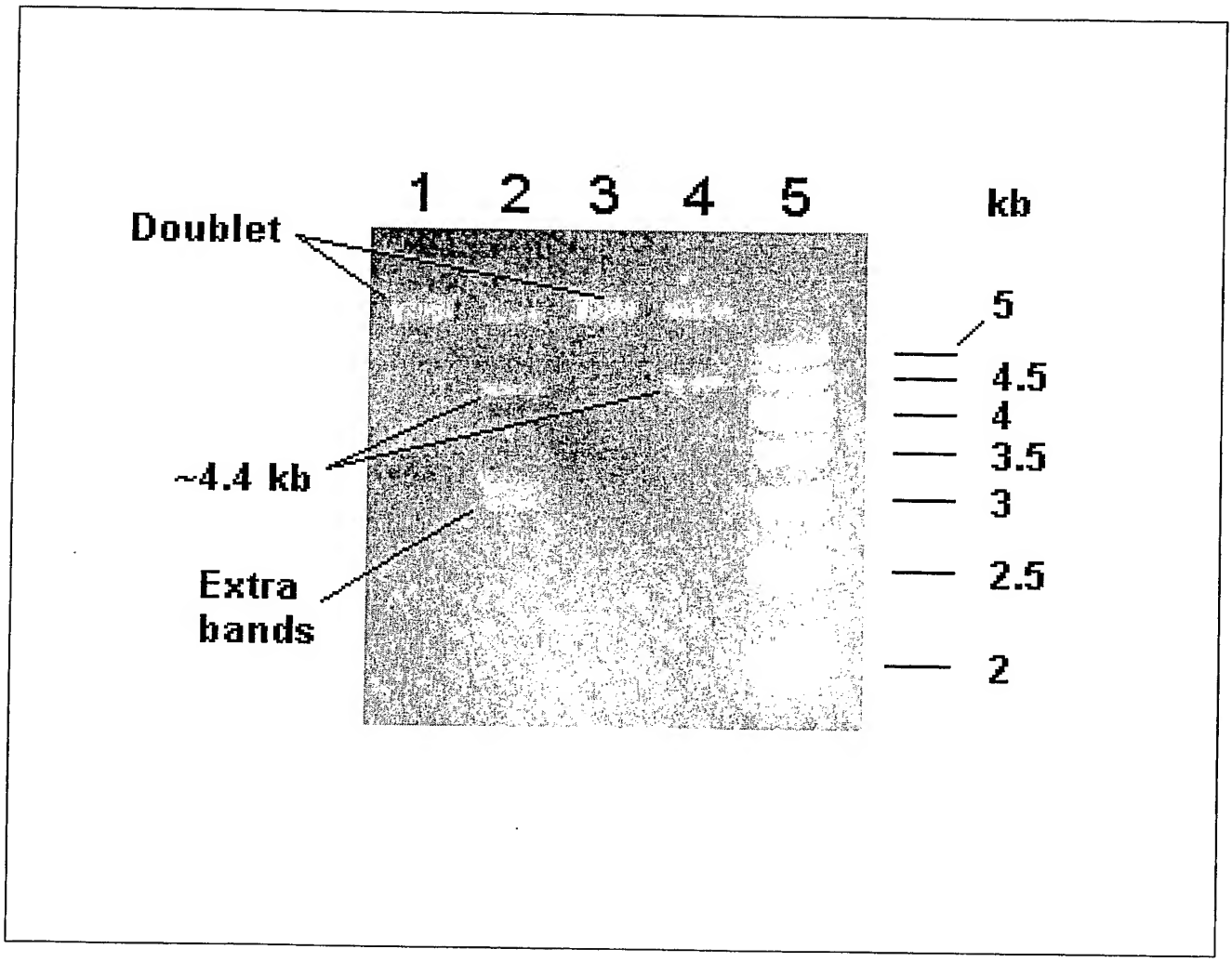


FIG. 7

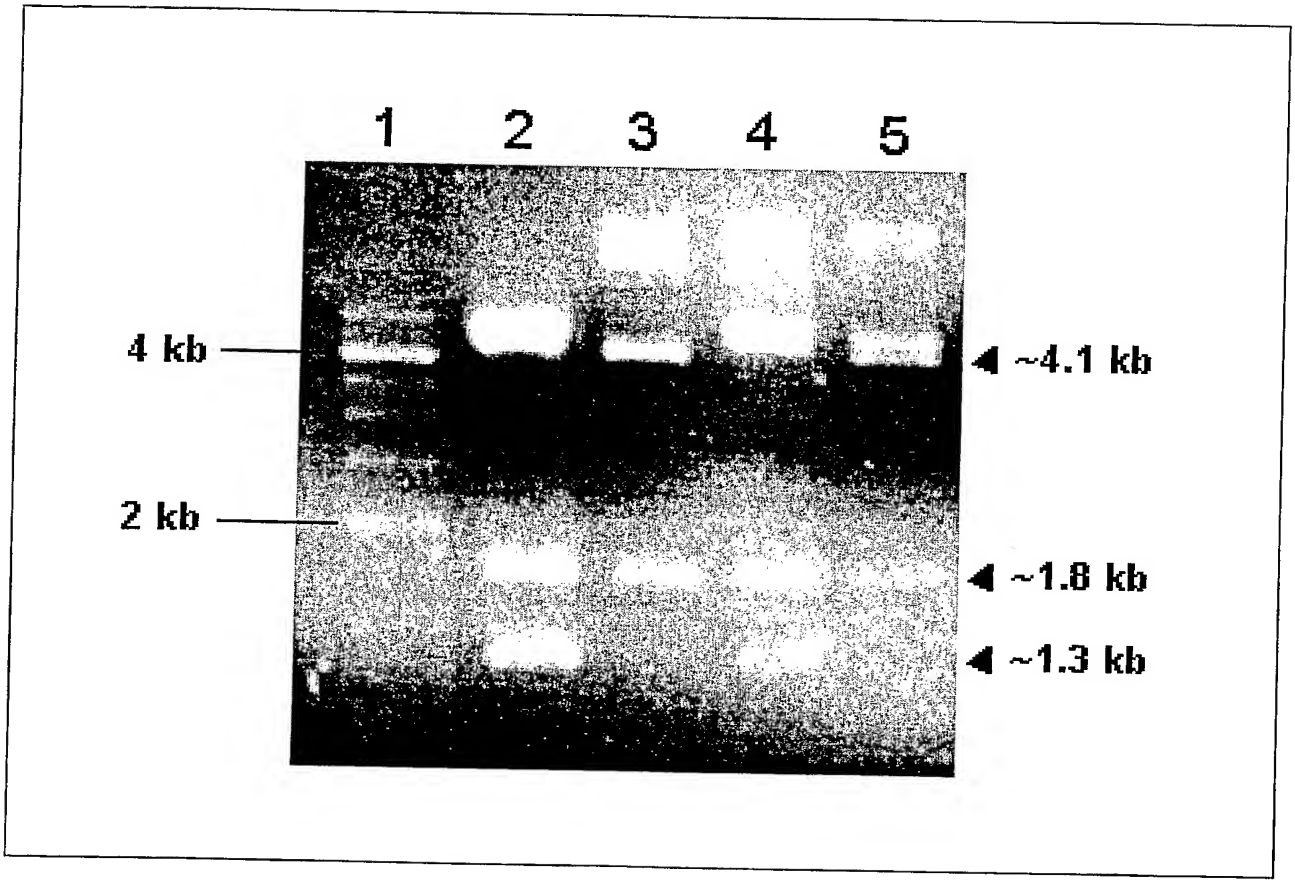


FIG. 8

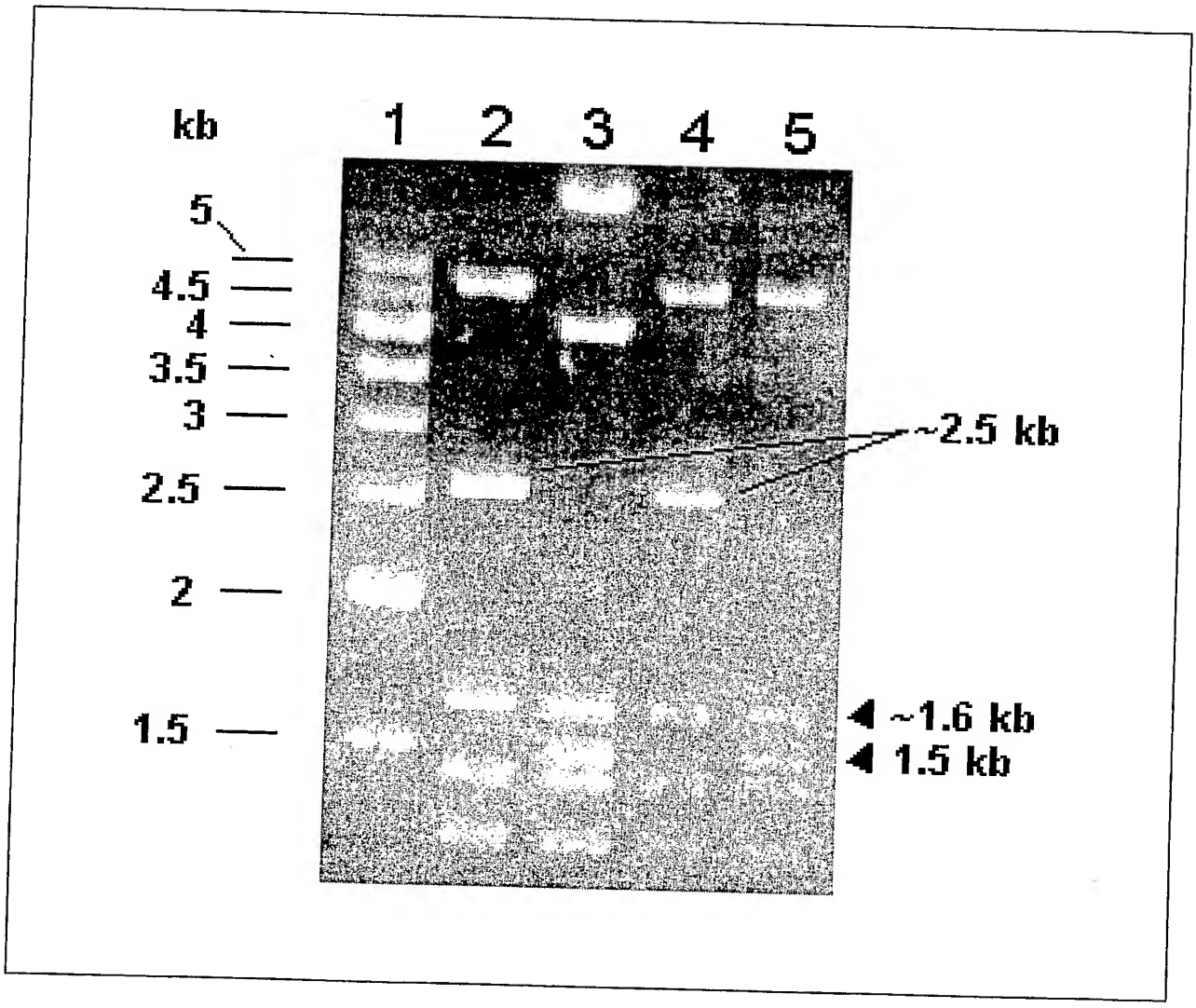


FIG.9

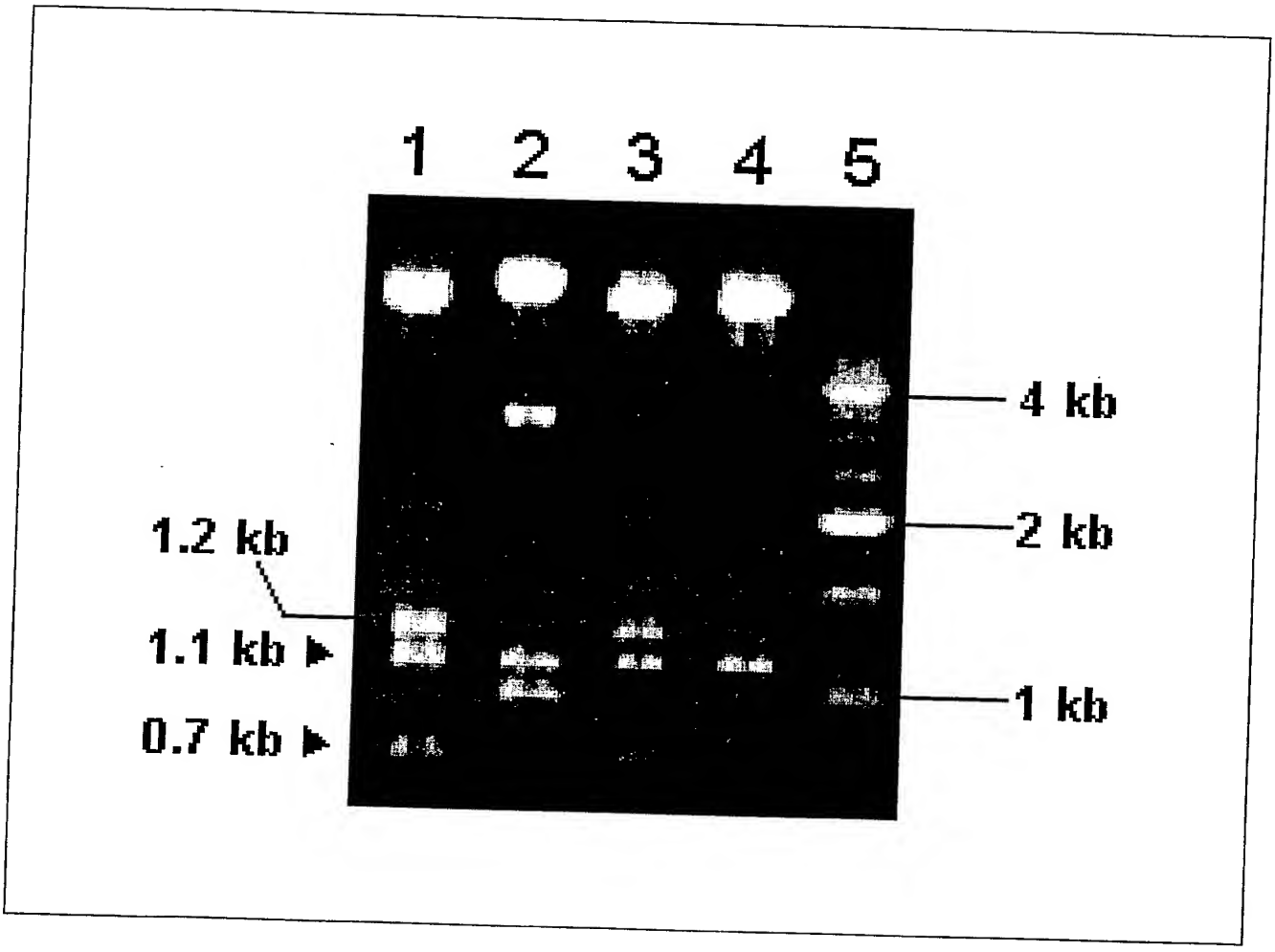


FIG. 10

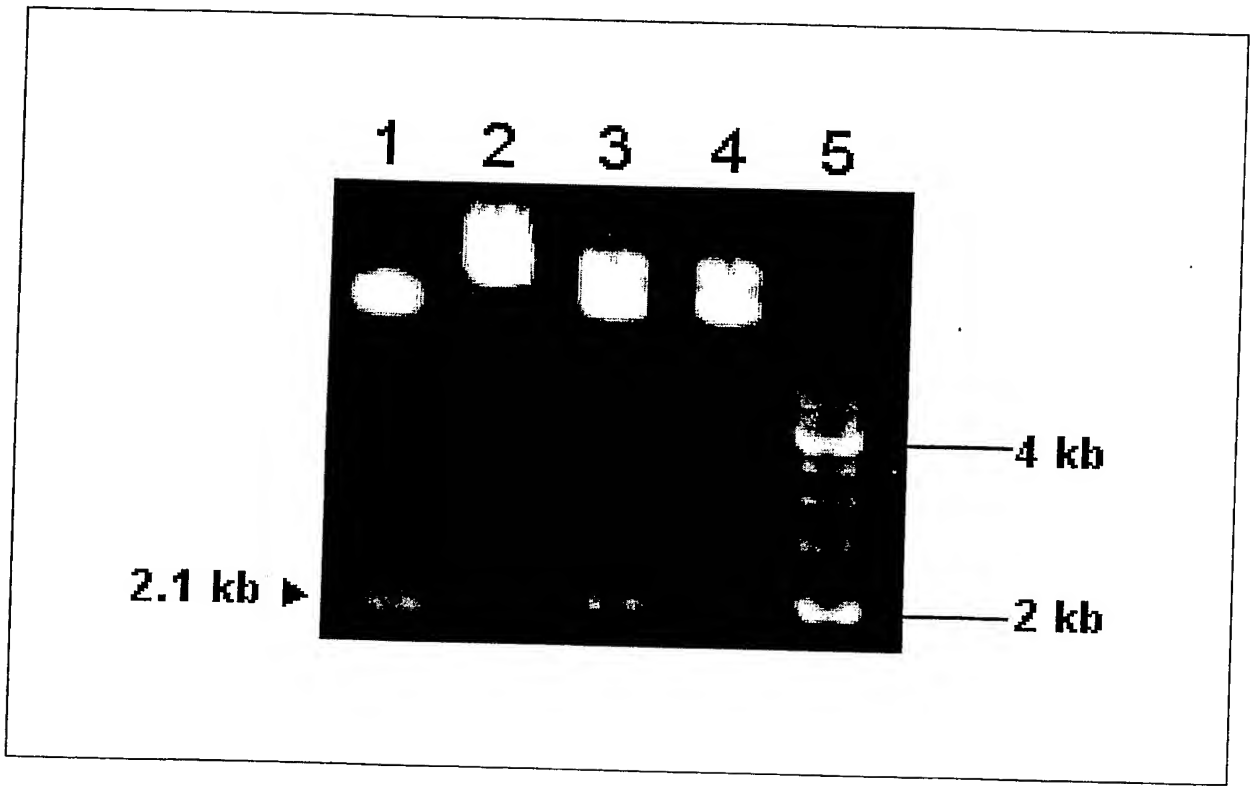


FIG. 11

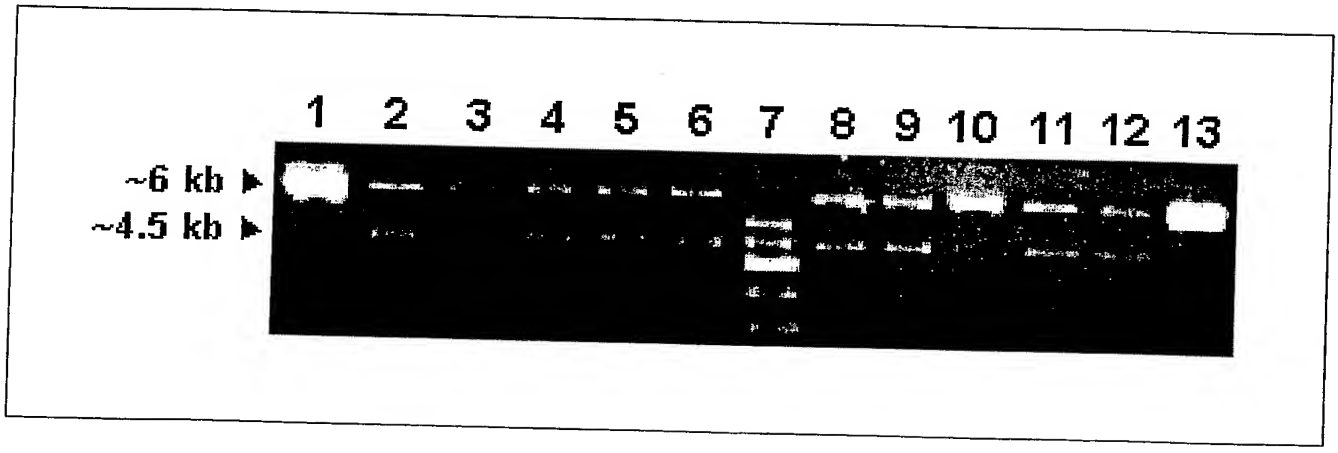


FIG. 12

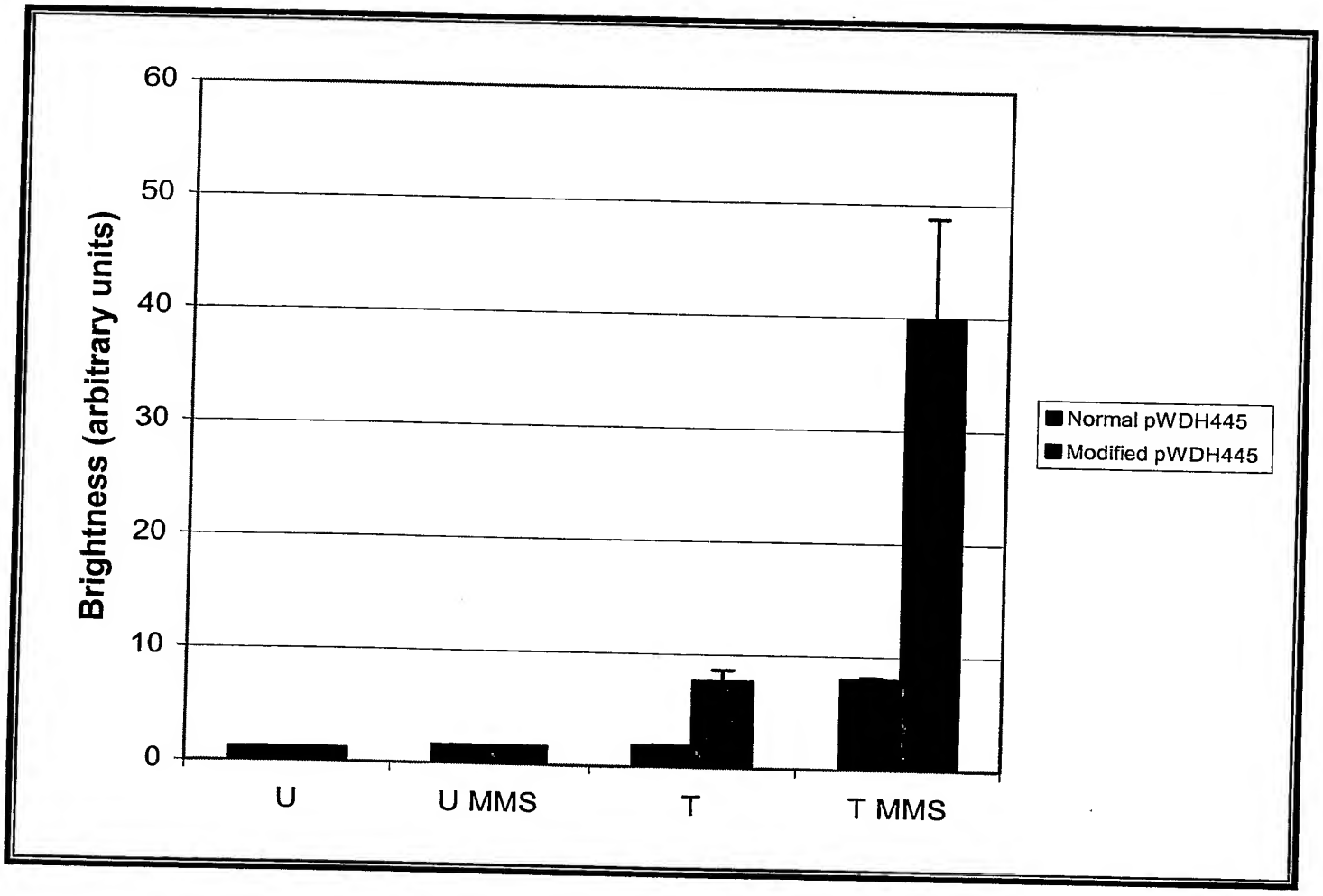

60
50
40
30
20
10
0
Brightness (arbitrary units)
U
U MMS
T
T MMS
Normal pWDH445
Modified pWDH445

FIG. 13

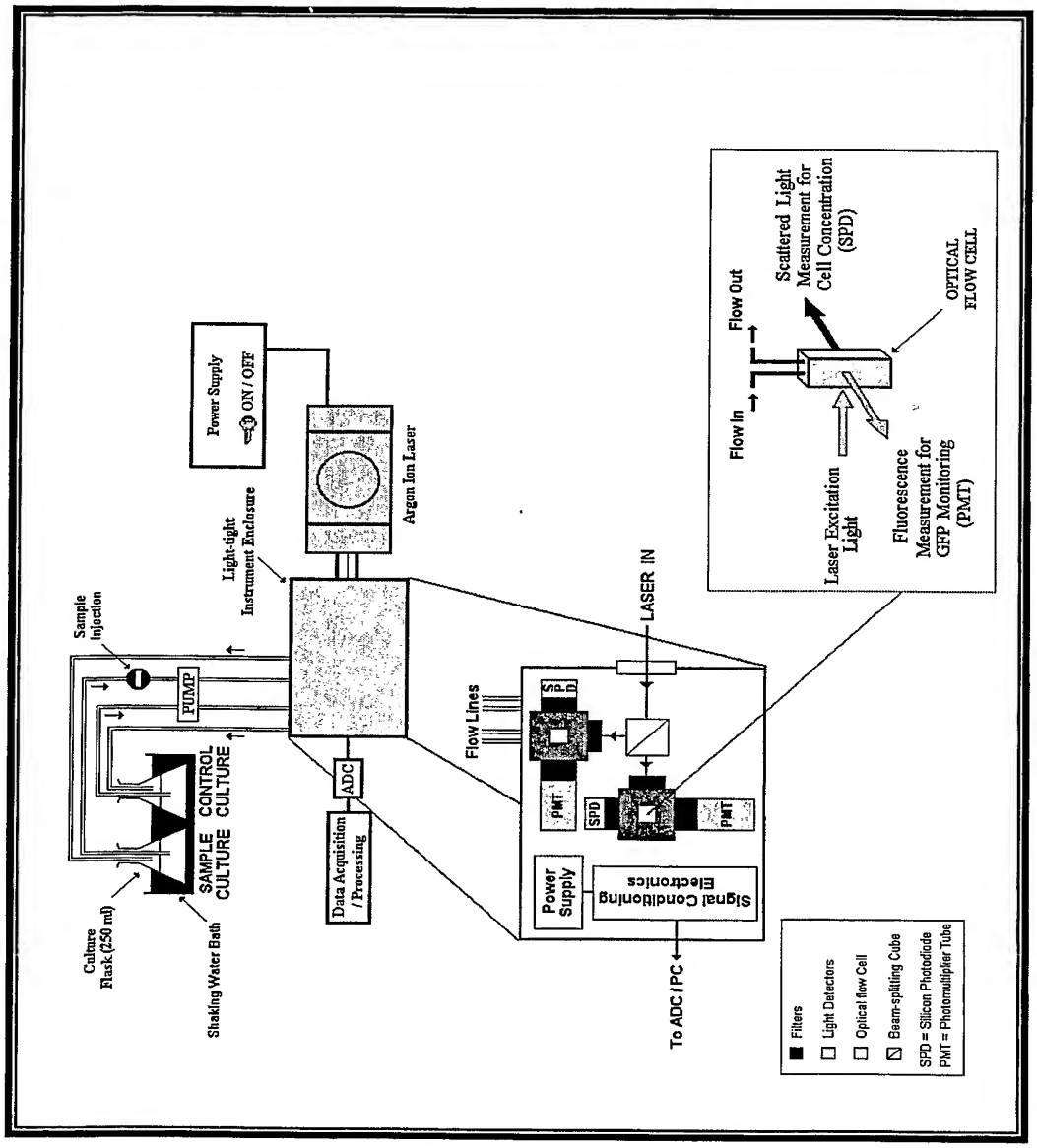

Power Supply
ON / OFF
Argon Ion Laser
Light-tight Instrument Enclosure
Sample Injection
PUMP
CONTROL CULTURE
SAMPLE CULTURE
Culture Flask (250 ml)
Shaking Water Bath
ADC
Data Acquisition / Processing
Flow Lines
LASER IN
S.P.D
PMT
SPD
PMT
Power Supply
Signal Conditioning Electronics
To ADC / PC
Flow Out
Scattered Light Measurement for Cell Concentration (SPD)
OPTICAL FLOW CELL
Flow In
Laser Excitation Light
Fluorescence Measurement for GFP Monitoring (PMT)
Filters
Light Detectors
Optical flow Cell
Beam-splitting Cube
SPD = Silicon Photodiode
PMT = Photomultiplier Tube

FIG. 14

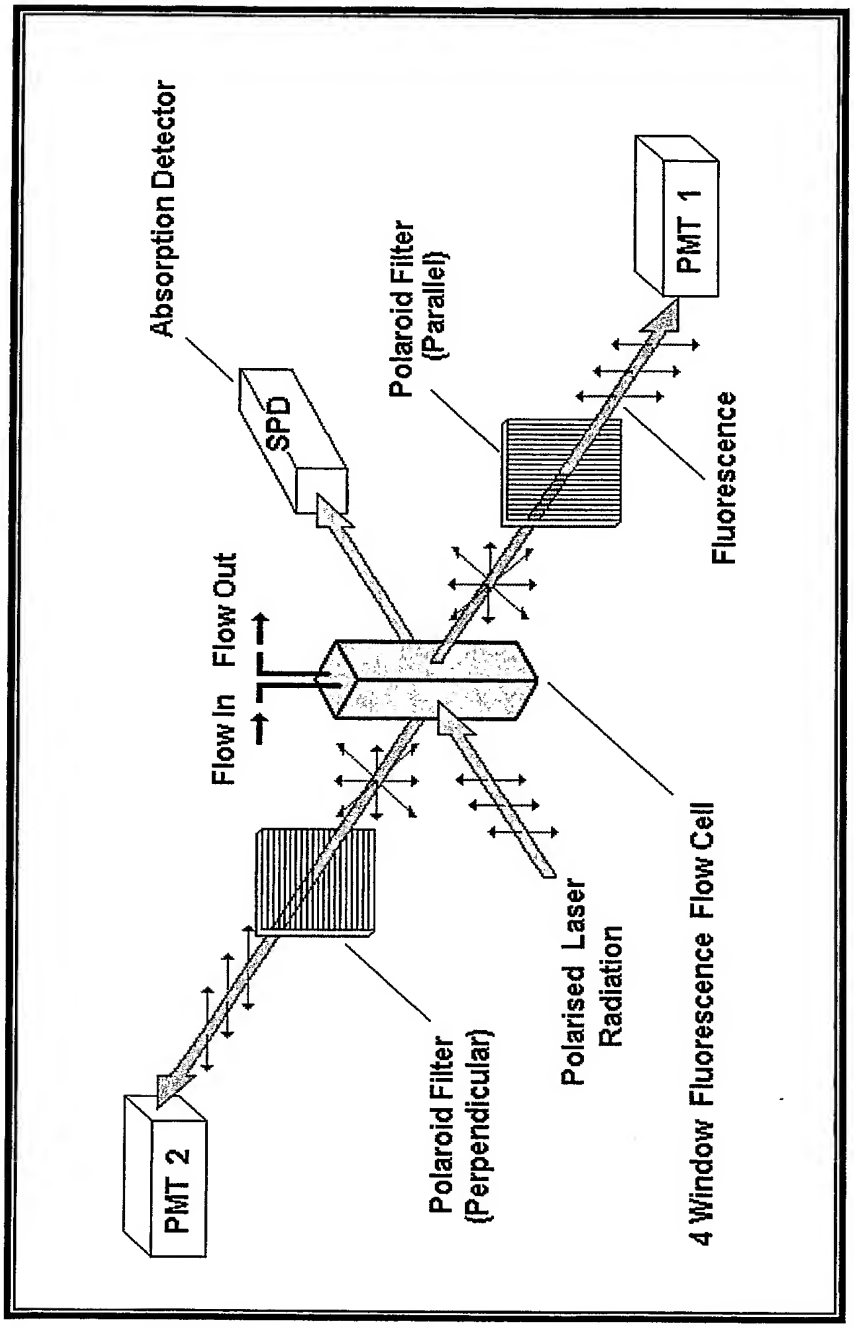
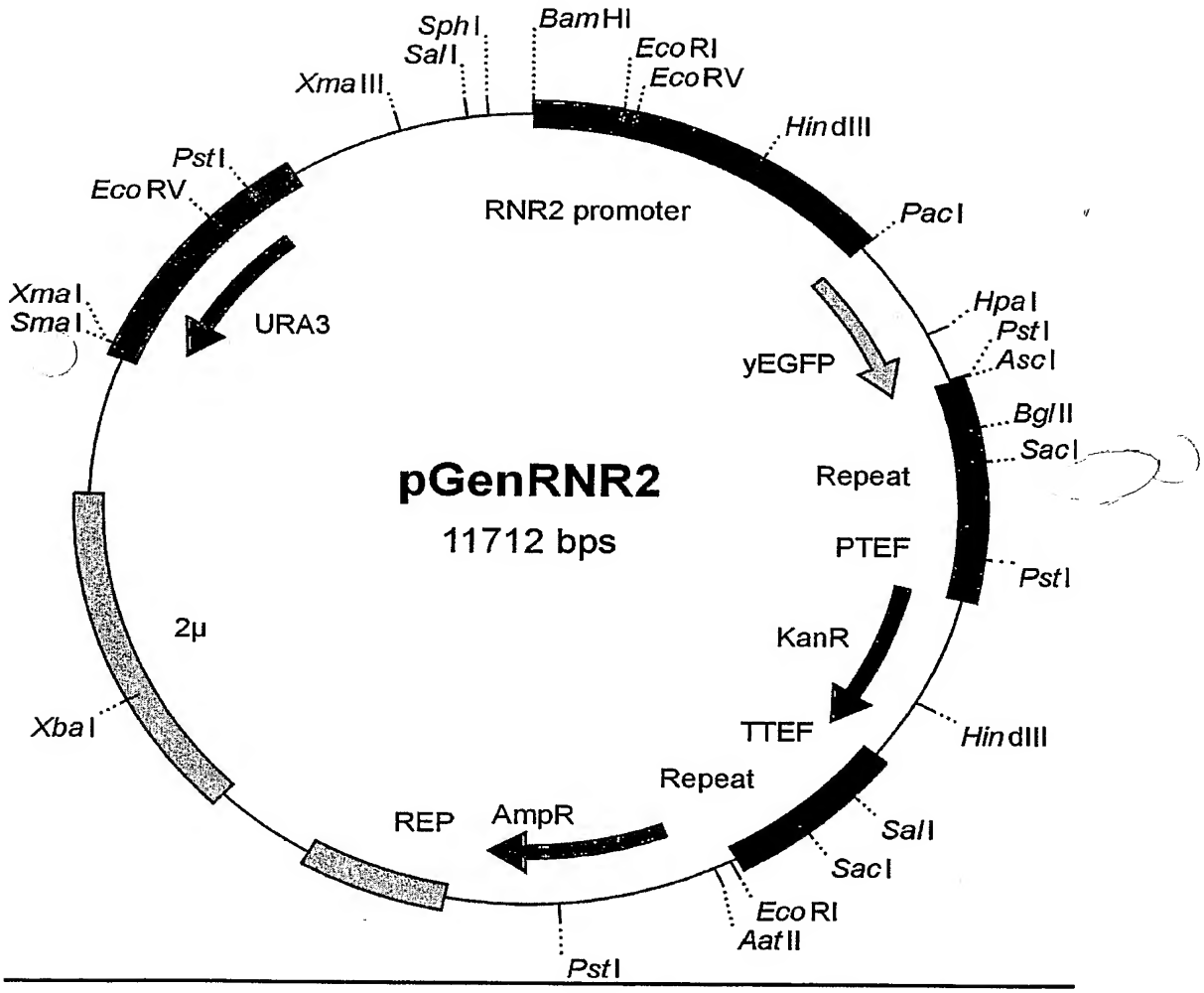

Absorption Detector
SPD
Polaroid Filter (Parallel)
PMT 1
Fluorescence
Flow In
Flow Out
Polaroid Filter (Perpendicular)
PMT 2
Polarised Laser Radiation
4 Window Fluorescence Flow Cell

FIG. 15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11712 bps
RNR2 promoter
URA3
yEGFP
Repeat
PTEF
KanR
TTEF
Repeat
AmpR
REP
2μ
Sph I
Sal I
Xma III
BamHI
EcoRI
EcoRV
HindIII
PacI
HpaI
PstI
AscI
BglII
SacI
PstI
HindIII
SalI
SacI
EcoRI
AatII
PstI
XbaI
XmaI
SmaI
EcoRV
PstI

FIG. 16

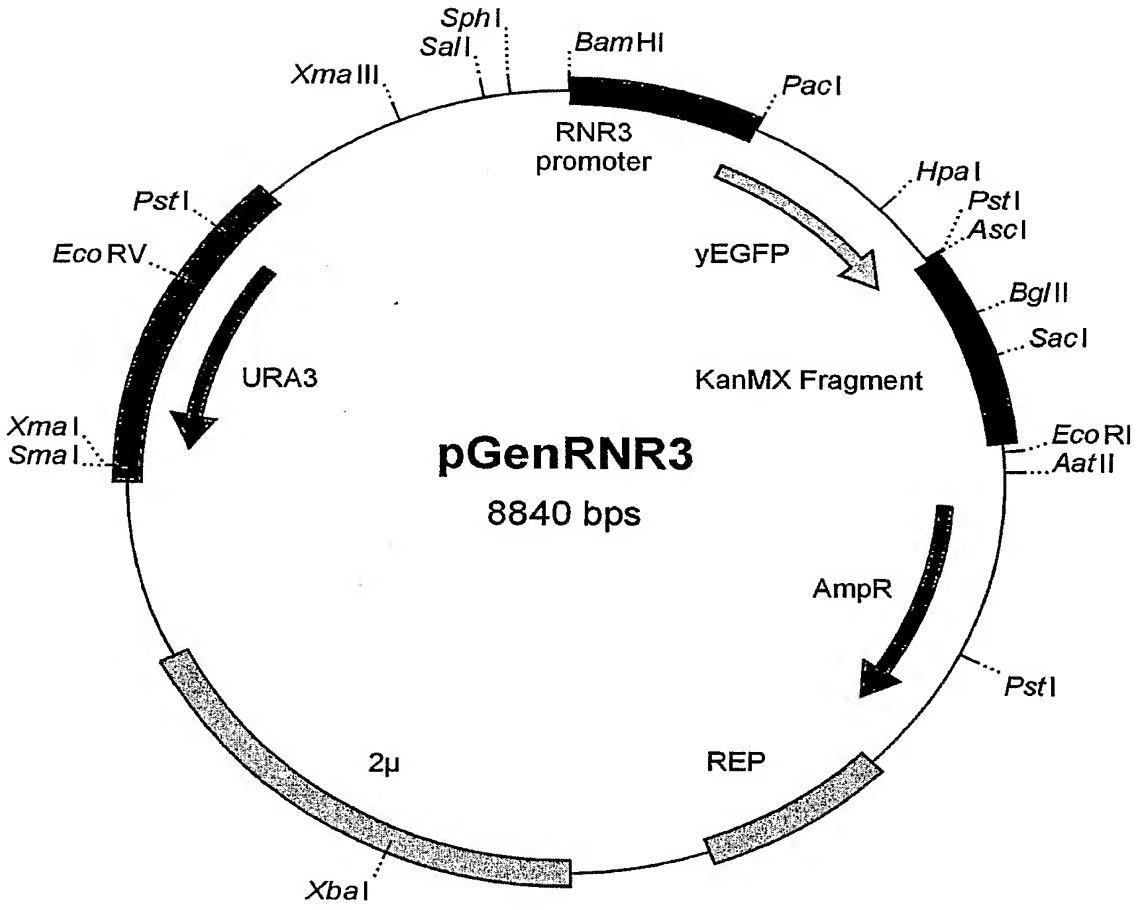
Sph I
Sal I
Bam HI
Xma III
Pac I
RNR3
promoter
Hpa I
Pst I
Asc I
Pst I
Eco RV
yEGFP
Bgl II
Sac I
URA3
KanMX Fragment
Xma I
Sma I
pGenRNR3
8840 bps
Eco RI
Aat II
AmpR
Pst I
2µ
REP
Xba I

FIG. 17

pGenRNR2

GGATCCGTACCTTCCAGCATTGTCCTTCTGAGAAAACAAAAATGGAAGATGTTGTGAAAATGCAGTAAGTGA
CAATAAGCTAGCGACTTTTCAATGATCGCAGATTAAGTATATATACACTTTAGGTCTCCTTCTCTTCTTTAC
TAGCTACGAAAAAACAAAGAAAAAAAAAACAAAATAGAGGGAAAAAAGATATACGTATAAAAATATGCTCCA
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CGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA
GTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTAT
GGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTC
CTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCC
GAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGC
ATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCC
AGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGC
GCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTG
TCAGAGGTTTTCACCGTCA

TCACCGAAACGCGCGAGGCAGAGCTTTGAAGAAAAATGCGCCTTATTCAATCTTTGCTATAAAAAATGGCCC
AAAATCTCACATTGGAAGACATTTGATGACCTCATTTCTTTCAATGAAGGGCCTAACGGAGTTGACTAATGT
TGTGGGAAATTGGAGCGATAAGCGTGCTTCTGCCGTGGCCAGGACAACGTATACTCATCAGATAACAGCAAT
ACCTGATCACTACTTCGCACTAGTTTCTCGGTACTATGCATATGATCCAATATCAAAGGAAATGATAGCATT
GAAGGATGAGACTAATCCAATTGAGGAGTGGCAGCATATAGAACAGCTAAAGGGTAGTGCTGAAGGAAGCAT
ACGATACCCCGCATGGAATGGGATAATATCACAGGAGGTACTAGACTACCTTTCATCCTACATAAATAGACG
CATATAAGTACGCATTTAAGCATAAACACGCACTATGCCGTTCTTCTCATGTATATATATATACAGGCAACA
CGCAGATATAGGTGCGACGTGAACAGTGAGCTGTATGTGCGCAGCTCGCGTTGCATTTTCGGAAGCGCTCGT
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AATGAAGAATCATCAACGCTATCACTTTCTGTTCACAAAGTATGCGCAATCCACATCGGTATAGAATATAAT
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CCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGT
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GTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTA
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GATTCCCGGGTAATAACTGATATAATTAAATTGAAGCTCTAATTTGTGAGTTTAGTATACATGCATTTACTT
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CACCCTCTACCTTAGCATCCCTTCCCTTTGCAAATAGTCCTCTTCCAACAATAATAATGTCAGATCCTGTAG
AGACCACATCATCCACGGTTCTATACTGTTGACCCAATGCGTCTCCCTTGTCATCTAAACCCACACCGGGTG
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TACCTGGGCCCACCACACCGTGTGCATTCGTAATGTCTGCCCATTCTGCTATTCTGTATACACCCGCAGAGT
ACTGCAATTTGACTGTATTACCAATGTCAGCAAATTTTCTGTCTTCGAAGAGTAAAAAATTGTACTTGGCGG
ATAATGCCTTTAGCGGCTTAACTGTGCCCTCCATGGAAAAATCAGTCAAGATATCCACATGTGTTTTTAGTA
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TCCTTCGTTCTTCCTTCTGCTCGGAGATTACCGAATCAAAAAAATTTCAAAGAAACCGGAATCAAAAAAAAG
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GACGCGATGGATATGTTCTGCCAAGGGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATTGATTGGCTCCA
ATTCTTGGAGTGGTGAATCCGTTAGCGAGGTGCCGCCGGCTTCCATTCAGGTCGAGGTGGCCCGGCTCCATG
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CGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTT
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GTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCG
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CCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATAT
AGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGA

FIG. 18

PGenRNR3

GGATCCAGAAACAAGAGAAGGTAACAAGCACATAAAAAATCAGCACATACGTACATACATAAGAATGAATCG
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TTTTTTTTTTTCGTGGTTGTCGCAGCAACGACACCTAGGCGCTGCTCAAAGGGGCAAAAACCCGGTTGCCAT
GGCGAGGACCAAACGACAAGATGGGAAAAAAACAATAGTCTATTGTTAAATCGTAATACTGTATTGTGAGAT
GCTGACGCGTTTCGTTTTTCGTGTCAGCGTTCTTTATATTGTTTCGTGTTCTGCTGCAAAACGTATATAAAC
GCACTGCTATTTTGCCTTCTTTTGCCTTCTTCCTTGCCTTTCTCTCATCTCATATCCAAGTTGAAATAAATA
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CCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAATTTATTTGTACTACTGGTAAATTGC
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TGAAACAACATGACTTTTTCAAGTCTGCCATGCCAGAAGGTTATGTTCAAGAAAGAACTATTTTTTTCAAAG
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CATGAGAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATG
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GGAGATGGCGGACGCGATGGATATGTTCTGCCAAGGGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATTG
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AGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTG
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AGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGT
CCCCCGGCCACGGGGCCTCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGA
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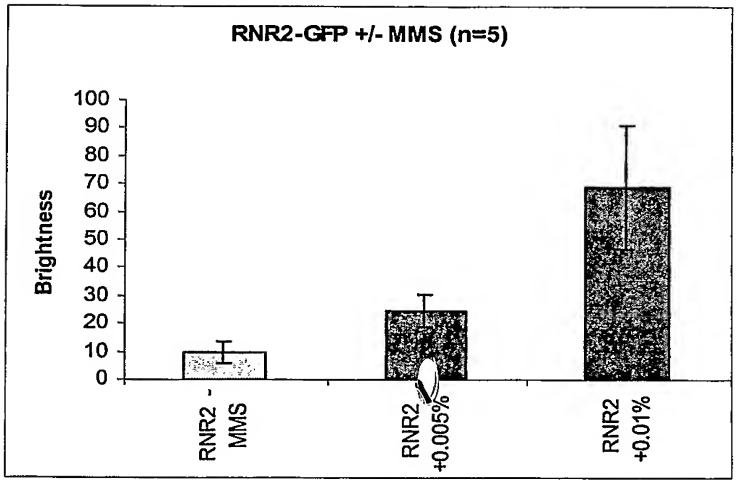
ATGCGTCCGGCGTAGA

FIG. 19

A


RNR2-GFP +/- MMS (n=5)
Brightness
100
90
80
70
60
50
40
30
20
10
0
RNR2 - MMS
RNR2 +0.005%
RNR2 +0.01%

B

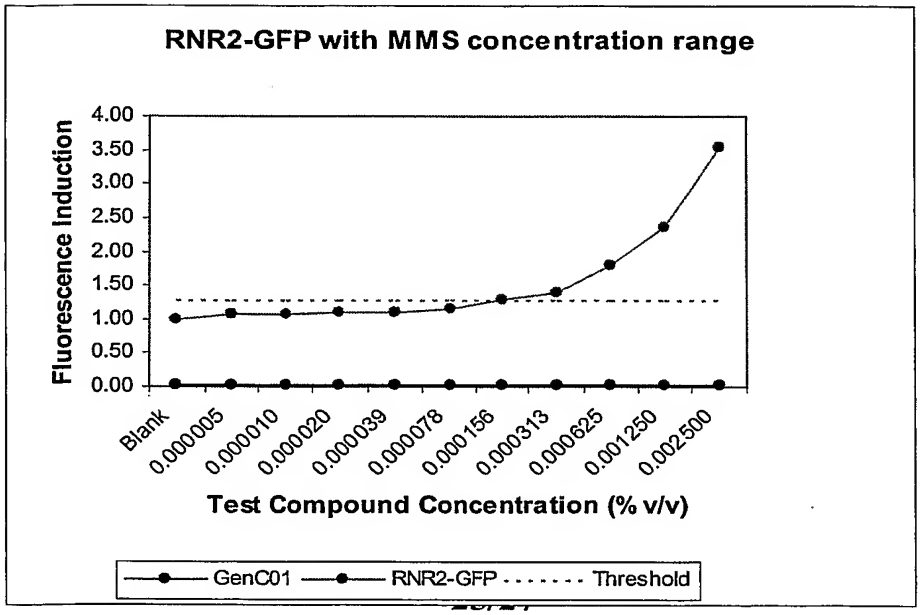
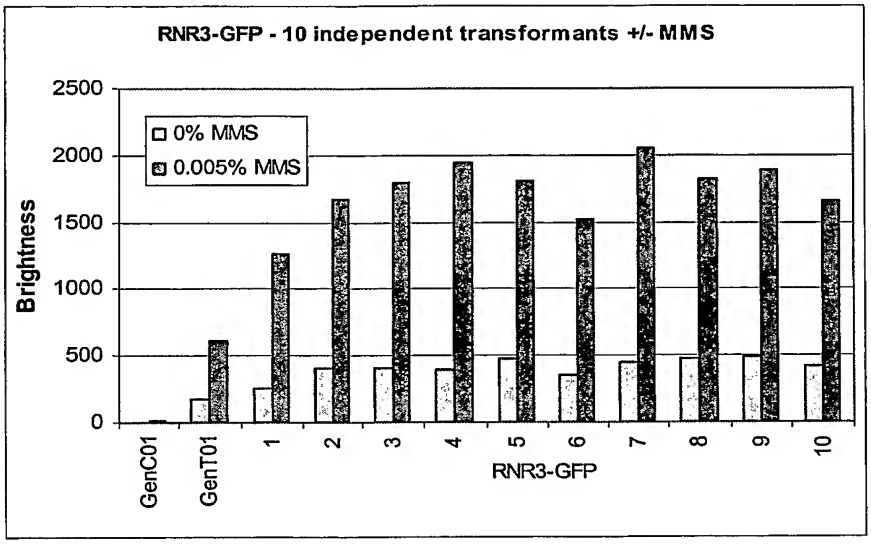

RNR2-GFP with MMS concentration range
Fluorescence Induction
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Blank
0.000005
0.000010
0.000020
0.000039
0.000078
0.000156
0.000313
0.000625
0.001250
0.002500
Test Compound Concentration (% v/v)
GenC01
RNR2-GFP
Threshold

FIG. 20

A


RNR3-GFP - 10 independent transformants +/- MMS
Brightness
2500
2000
1500
1000
500
0
0% MMS
0.005% MMS
GenC01
GenT01
1
2
3
4
5
6
7
8
9
10
RNR3-GFP

B

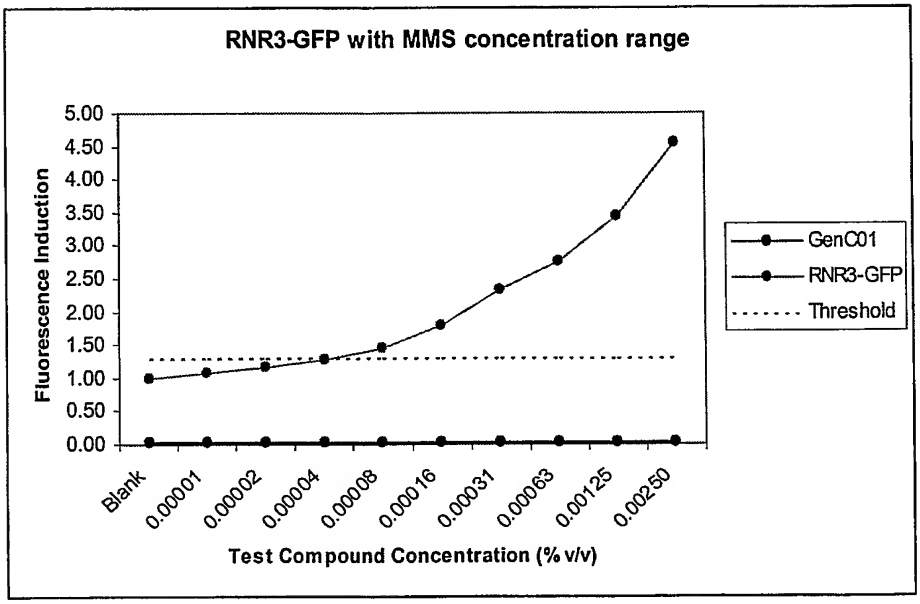

RNR3-GFP with MMS concentration range
Fluorescence Induction
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
GenC01
RNR3-GFP
Threshold
Blank
0.00001
0.00002
0.00004
0.00008
0.00016
0.00031
0.00063
0.00125
0.00250
Test Compound Concentration (% v/v)

FIG. 21

Greenrack loading sequence

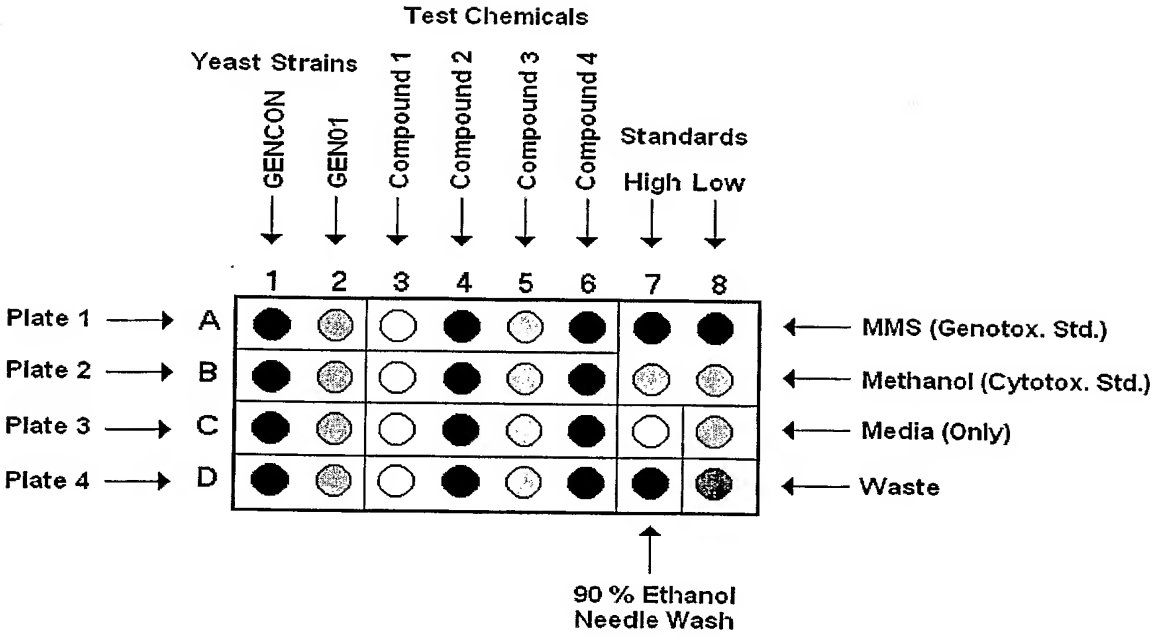


FIG. 22

Microplate layout